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Extraction Method Development and *in vivo* and *in vitro* Toxicity Studies of the Etiologic Agent of Avian Vacuolar Myelinopathy

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EXTRACTION METHOD DEVELOPMENT AND *IN VIVO* AND *IN VITRO* TOXICITY STUDIES OF THE ETIOLOGIC AGENT OF AVIAN VACUOLAR MYELINOPATHY

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ABSTRACT

Avian vacuolar myelinopathy (AVM) is a neurological disease affecting birds in the southeastern United States. The cause of the disease has not yet been determined but is believed to be a naturally produced toxin and is associated with aquatic vegetation. Current research on AVM is limited to *in vivo* studies utilizing whole tissue or vegetative samples. The objectives of this research were to develop extraction methods for isolating the putative AVM toxin from vegetative samples and to develop an *in vitro* bioassay for detection and study of the toxin. Samples of vegetation were collected from reservoirs known to be affected by AVM and confirmed to contain the toxin by mallard bioassay. The collected vegetation was then extracted with a series of solvents and the crude extracts were re-introduced to a mallard model to confirm the presence or absence of the AVM agent. All birds administered a methanol extract developed characteristic AVM lesions, indicating that methanol is a suitable solvent for AVM toxin extraction. Additional crude extracts were produced from vegetation collected at AVM-affected reservoirs as well as reservoirs with no known history of AVM and evaluated for their *in vitro* toxicity using established cell lines. Extracts produced from vegetation collected at AVM sites induced a significant cell cycle arrest in C6 glioma cells, while AVM-negative extracts induced only mild effects on this endpoint. To further evaluate this *in vitro* assay for its ability to detect the AVM toxin, as well as continue the development of toxin extraction

methods, fractions were produced from a crude methanol extract and evaluated for toxicity by the cell cycle assay as well as an *in vivo* chicken bioassay. The fractionation methods were successful in isolating the *in vitro* toxicity, but the presence of the AVM toxin could not be confirmed in these fractions due to inconclusive *in vivo* results. Further testing will be necessary to determine if the observed cell cycle arrest is due to the etiologic agent of AVM as well as to advance the process of AVM toxin isolation and characterization, but this research has providing promising tools for future AVM investigations.

DEDICATION

This dissertation is dedicated to my parents, Bill and Vivian Wiley; my grandmother, Dora Wiley; and my best friend and soon-to-be husband, Bran Cromer. Your support has meant the world to me. I love you all so much.

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PREFACE

This dissertation was written in journal style and organized into five chapters, preceded by a general introduction and followed by a conclusions section. The chapters represent different research projects and each is comprised of introduction, materials and methods, results, discussion, and literature cited sections. Each chapter is intended for separate publication; therefore, repetition is present in some sections.

INTRODUCTION

Avian vacuolar myelinopathy (AVM) is a neurological disease primarily affecting bald eagles (*Haliaeetus leucocephalus*) and American coots (*Fulica americana*) in the southeastern U.S. AVM was first observed during the winter of 1994-1995 and has been documented in five states: Arkansas, Georgia, North Carolina, South Carolina, and Texas. Since 1994, AVM has been responsible for the deaths of over 100 bald eagles and estimates of over a thousand coots. Despite extensive investigations, the cause of AVM remains unknown, though all information gathered to date suggests a toxin, most likely of natural origin.

AVM is characterized and diagnosed by a spongy degeneration of the white matter of the central nervous system (CNS). Multiple vacuoles of varying severity are seen throughout the white matter of the brain and spinal cord, being particularly prominent in the optic tectum (Thomas et al., 1998). Birds with AVM may display clinical signs of neurological impairment, including difficulty in swimming, flying, and/or walking. Not all birds with AVM lesions display these signs, and clinical recovery has been documented despite the persistence of lesions (Larsen et al., 2002).

The first documented cases of AVM occurred during the winter of 1994-1995 at DeGray Lake, Arkansas, USA (Thomas et al., 1998). During this event, 29 bald eagles were found either dead or dying, and those still alive displayed signs of neurological impairment. Two winters later, 26 eagles died at DeGray Lake and nearby Lakes Ouachita and Hamilton. Wintering American coots were

observed with similar signs of impairment during both of these events, though only a small number were found dead. Thorough diagnostic evaluations of eagles and coots were conducted by the National Wildlife Health Center, Madison, Wisconsin, USA. No consistent gross abnormalities were seen and no infectious disease agents were discovered. In addition, no significant concentrations of any toxins or toxicants were detected. The only consistent finding was the presence of microscopic vacuoles throughout the white matter of the CNS. Electron microscopy confirmed the vacuoles were formed by separation of the myelin lamellae at the intraperiod line, a condition known as intramyelinic edema (Thomas et al., 1998). This condition can be caused by several known chemicals, including triethyltin, hexachlorophene, bromethalin, and others, though none of these compounds were detected in tissues.

AVM was first documented outside of Arkansas during the winter of 1997-1998, when affected coots were observed at Woodlake (Lake Surf), North Carolina and Lake Juliette, Georgia. The following winter, additional affected coots were observed at these sites, as well as at Lake Ouachita, Arkansas and three sites in South Carolina: Lake Murray, J. Strom Thurmond Lake (JSTL), and Par Pond, a reservoir at the Savannah River Site (SRS). In addition, Lake Juliette, Woodlake, JSTL, and SRS each had one eagle mortality. AVM was first documented in waterfowl during the winter of 1998-1999, with a low incidence of AVM in mallards (*Anas platyrhynchos*), ring-necked ducks (*Aythya collari*), and buffleheads (*Bucephala albeola*) at Woodlake (Augspurger et al., 2003). The largest eagle mortality events outside of Arkansas occurred at JSTL during the

winters of 2000-2001 and 2001-2002, during which 23 eagles died. Several more species have also been documented with AVM, including Canada geese (*Branta canadensis*), great-horned owl (*Bubo virginianus*), and killdeer (*Charadrius vociferus*) (Fischer et al., 2002).

In recent years, eagle mortalities continue to be observed and AVM-positive coots are observed each year at sites where active monitoring is conducted (Fischer et al., 2006). In addition, several more sites of AVM mortalities have been documented in recent years, including several small residential ponds in Georgia and North Carolina, and a small farm pond in South Carolina (Wilde et al., 2005; Fischer, unpubl. data). These sites demonstrate that AVM is not restricted to large reservoirs and may be undetected in smaller systems.

While the cause of AVM remains elusive, evidence gathered to date suggests a toxin of natural origin. The lack of inflammatory response in tissues and inability to detect any infectious disease agents makes the probability of a bacterial or viral infection very low. In addition, the disease has not been shown to be transmissible by direct contact with affected birds (Larsen et al., 2003). The pathology of AVM suggests an acutely toxic compound, since the lesions manifest quickly without any systemic involvement (Thomas et al., 1998). The nature of AVM epornitics also indicates a toxin as the source of disease. Rocke et al. (2002) conducted a sentinel study in which healthy coots and mallards were released over a four-year period onto Woodlake. Results showed that AVM is site-specific, can have a quick time of onset (as early as 5 days post-release), and

is seasonal, with AVM events occurring during the late fall to early winter and lasting up to several months.

The search for the toxic agent has proven challenging. As mentioned, extensive toxicological tests were conducted on coot and eagle tissues during the first AVM epizootics (Thomas et al., 1998). Various tissues were analyzed for a wide spectrum of organic and inorganic compounds, but no significant concentrations were detected. In a separate study, coot tissues and sediments from AVM reservoirs and control sites were analyzed for lipophilic organic compounds in hopes of finding a compound unique to AVM sites (Dodder et al., 2003). Despite the presence of detectable contaminants, no compound could be singled out as a probable suspect.

Although the causative agent has not yet been discovered, it is now evident that AVM is acquired orally, through consumption of affected food items. In the first study to reproduce AVM in the laboratory, Fischer et al. (2003) demonstrated transmission from AVM-affected coot tissue to red-tailed hawks (*Buteo jamaicensis*), establishing the link between coots and eagles in the wild. During coot migrations, which generally coincide with AVM epornitics, these water birds are a major food source for bald eagles.

With evidence that AVM is acquired by ingestion, the food sources of coots in these reservoirs became a focus of research. All known AVM reservoirs contain one or more of the exotic aquatic plants hydrilla (*Hydrilla verticillata*), Brazilian elodea (*Egeria densa*), or Eurasian watermilfoil (*Myriophyllum spicatum*). These species cover extensive acreage in the reservoirs and serve as

the major food item of wintering coots. The first study to attempt induction of AVM in waterfowl through ingestion of this aquatic vegetation was unsuccessful. In that study, mallards were orally gavaged with hydrilla from an AVM reservoir for seven days (Larsen et al., 2003). Sediment and water from the same reservoir were also examined as potential sources. No mallards developed lesions, though the authors suggest that exposure duration and quantity of material consumed may have been inadequate to induce the disease, or the vegetation could have been collected at a time when the AVM agent was not present.

Three independent studies have now confirmed that coots and waterfowl do acquire AVM though ingestion of aquatic vegetation. These studies induced AVM in mallards and domestic chickens (*Gallus domesticus*) by ingestion of hydrilla and associated materials collected from JSTL during an AVM epizootic (Birrenkott, 2003; Birrenkott et al., 2004; Lewis-Weis et al., 2004; Rocke et al. 2005). The studies allowed the birds to feed *ad libitum*, with exposures ranging from 23 days to seven weeks. Hydrilla does not appear to be the causative agent of AVM, as it does not occur at all AVM sites and it is found in other areas where AVM has not been documented. It is thought, rather, that something associated with aquatic vegetation may be the cause.

Hydrilla collected from JSTL that induced AVM in the mallard laboratory study (Birrenkott et al., 2004) contained large quantities of a novel cyanobacteria species. Colonies of this species, a previously uncharacterized member of the Order Stigonematales (Williams et al., 2007), covered 50-90% of the leaf surface of the hydrilla (Birrenkott et al., 2004). Surveys of cyanobacterial epiphytes

revealed the consistent presence of this Stigonematalan species at all known sites of AVM epizootics (Wilde et al., 2005). The species was also found at a few sites where AVM has not been documented, though the relative abundance at these sites was significantly less than sites of AVM epornitics (Wilde et al., 2005). Researchers have postulated that this species produces a toxin responsible for AVM. The novel Stigonematalan species has been successfully cultured in the laboratory and genetic and physiological analyses have begun (Habrun, 2004). A preliminary feeding trial in which Stigonematalan cultures were fed by gavage to American coots produced no definitive lesions in the treatment birds (Wilde, unpubl. data). This does not disprove the hypothesis that the cyanobacteria are the producer of the toxin. The cyanobacterial cultures may not have been actively producing the toxin at the time of feeding, or the birds may not have been exposed to sufficient concentrations for the disease to develop. Cultures of the cyanobacteria are currently being grown under differing nutrient and temperature regimes, and these cultures will be used in future feeding trials.

Much research is still needed in the study of AVM, in addition to determining the cause and source of the disease. The pathology, range of susceptible species, implications for wildlife populations, and management strategies all need to be further explored. Areas of potential research on the pathology of AVM include mechanism of action, exposure time and concentration necessary to produce the disease, possibility of recovery, and acute versus chronic exposure effects. It is unknown why some birds develop clinical neurological signs and others do not. Clinical recovery has been shown in coots after being

taken into captivity, though lesions were still present upon necropsy 65 days later (Larsen et al., 2002). It is unknown whether or not the lesions can heal. In addition, there have been several reports of birds displaying clinical signs consistent with AVM without the presence of lesions upon necropsy (Lewis-Weis et al., 2004; Rocke et al., 2005).

The list of species known to be susceptible to AVM is currently confined to birds, but susceptibility of other classes of organisms should be further explored. Lesions have never been confirmed in mammals during AVM epizootics, though there has been anecdotal evidence of possible clinical signs in a beaver (*Castor canadensis*) (Lewis-Weis et al., 2004). Histological analysis of brains from beavers, raccoons (*Procyon lotor*), and a gray fox (*Urocyon cinereoargenteus*) collected at J. Strom Thurmond Lake revealed no abnormalities, despite the presence of coot tissues in the intestinal contents of one raccoon and the fox (Fischer et al., 2006). Swine failed to develop lesions when fed AVM-affected coot tissue in a laboratory study, though the authors acknowledge that the duration of exposure and quantity of ingested material should be explored further before concluding that mammals are not susceptible (Lewis-Weis et al., 2004). Mice fed hydrilla collected during AVM events also failed to develop lesions (Rocke et al., 2005; Birrenkott, unpubl. data); however, these animals did not appear to consume much, if any, of the vegetation in one study (Birrenkott, unpubl. data). It is also possible that the vegetation did not contain the causative agent in sufficient quantity to induce disease.

The impact of AVM to wildlife populations is largely unknown. Birrenkott (2003) examined the impact of AVM on South Carolina and Georgia's bald eagle population, concluding that AVM has significantly affected local populations. Since eagles in these states are non-migratory and their breeding seasons coincide with AVM epizootics, AVM could pose a significant risk to statewide populations if it should spread to other reservoirs. Impacts to coot and waterfowl populations are beginning to be explored.

Management of AVM epizootics is also an area that needs to be further addressed. Considerations for management will ultimately depend upon determination of the source of the disease. However, the knowledge that AVM is associated with aquatic vegetation, in particular invasive plants, has given researchers and managers an area of focus. Exotic aquatic weeds have been impacting the nation's waterways since the late 1800s, and management of these plants is still a major area of research today (Cofrancesco, 1998). Various mechanical, chemical, and biological control methods have been used, with varying degrees of success. Biological control, which involves the introduction and release of exotic insects, predators, or pathogens, is presently the predominant method of weed control and subject of much current research (McFayden, 1998). The knowledge that AVM is associated with noxious weeds adds an additional factor of consideration for aquatic plant managers working in AVM reservoirs. Researchers are currently studying sterile grass carp (*Ctenopharyngodon idella*) as a potential hydrilla control agent in AVM reservoirs. Grass carp have been used to control aquatic weeds in the U.S. since the 1960s, with much success

(Masser, 2002). Current research is focused on whether or not carp are affected by the AVM toxin, and whether they could be a potential vector of the disease to predators. Similar considerations and study should be made with other weed control agents currently used in reservoirs where AVM has been documented.

The purpose of my research was to examine the putative toxin responsible for AVM and it consists of two major objectives. The first objective was to develop an extraction procedure for isolating the toxin from its environmental matrix, i.e. aquatic vegetation and associated epiphytes. Without an extraction procedure, AVM researchers are limited to *in vivo* studies involving whole tissue or vegetative samples. While such studies can be useful, they are limited in terms of the information they can provide. Research on toxin chemistry, and eventually toxin isolation and identification, necessitates the isolation of the putative toxin from the environmental matrix. This involved first obtaining vegetation known to contain the toxin (Chapter 1) then developing and testing crude extracts produced from this material (Chapter 2). Once a suitable method was developed for extracting the AVM toxin from vegetation, further fractionation of the crude extract was conducted in an attempt to produce a more purified extract (Chapter 4).

The second major objective of this research was to develop an *in vitro*, cell-based assay for detection of the AVM toxin. An *in vitro* model will allow for rapid and sensitive detection of the toxin and can thus be used to guide toxin fractionation and isolation studies. In addition, such assays can be used to study the biochemistry and pathology of the toxin. Established cell lines were exposed

to extracts produced from both AVM-positive and AVM-negative hydrilla and toxicity was assessed by examining several endpoints, including cytotoxicity, mechanism of cell death (apoptosis v. necrosis), morphological and cytoskeletal changes, and cell cycle disruption (Chapter 3). Cell cycle disruption was observed in one cell line and this assay was then used to track toxicity in the extract fractions produced in Chapter 4.

In addition to the two main objectives, mammalian susceptibility was explored using a mouse model (Chapter 5). Mice were exposed to the confirmed AVM-positive extracts produced in Chapter 2 by oral gavage, providing a better assessment of AVM susceptibility than previous mammalian toxicity studies.

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CHAPTER 1

INVESTIGATION OF THE LINK BETWEEN AVIAN VACUOLAR MYELINOPATHY AND A NOVEL SPECIES OF CYANOBACTERIA THROUGH LABORATORY FEEDING TRIALS

Introduction

Avian vacuolar myelinopathy (AVM) is a neurological disease affecting bald eagles (*Haliaeetus leucocephalus*), American coots (*Fulica americana*), and other birds in the southeastern U.S. First observed in 1994, the cause of AVM remains elusive despite extensive investigation, although the pathology of the disease and epizootiologic studies suggest a toxin (Thomas et al., 1998; Rocke et al., 2002).

AVM is characterized by microscopic vacuolization of the white matter of the central nervous system, resulting from a separation of the myelin lamellae at the intraperiod line (Thomas et al., 1998). This pathology is consistent with intramyelinic edema, a condition that can be caused by several known compounds, including triethyltin, hexachlorophene, and bromethalin (van Gemert and Killeen, 1998). No significant concentrations of any of these compounds or various other environmental contaminants have been detected in tissues or environmental samples from AVM mortality events. In addition, there has been no evidence to link AVM to infectious agents (Thomas et al., 1998; Larsen et al., 2003).

Epidemiological investigations and sentinel studies have established that AVM is site-specific, seasonal in occurrence, and can have a rapid onset (Rocke et al., 2002). AVM generally occurs during late fall and early winter and the onset of disease can occur as quickly as five days after exposure to the causative agent. In addition to bald eagles and American coots, several other avian species have been diagnosed with AVM, including mallards (*Anas platyrhynchos*), ring-necked ducks (*Aythya collaris*), buffleheads (*Bucephala albeola*), Canada geese (*Branta canadensis*), great-horned owls (*Bubo virginianus*), and a killdeer (*Charadrius vociferus*) (Fischer et al., 2002; Augspurger et al., 2003). Birds with AVM may display clinical signs, including difficulty or inability to walk, swim, and/or fly and general ataxia. Not all birds with AVM lesions display these signs, thus microscopic evaluation of brain tissue is necessary for diagnosis.

Recent studies have established that AVM is acquired through ingestion and transferred through the food chain. Transfer of AVM from water birds to raptors was demonstrated when captive red-tailed hawks (*Buteo jamaicensis*) developed AVM after ingesting AVM-affected coot tissue in the laboratory (Fischer et al., 2003). AVM was experimentally induced in both mallards and domestic chickens (*Gallus domesticus*) through ingestion of aquatic vegetation (*Hydrilla verticillata* and associated materials) from AVM affected reservoirs (Birrenkott et al., 2004; Lewis-Weis et al., 2004).

Researchers are now examining a possible link between AVM and a species of cyanobacteria associated with the aquatic vegetation. Surveys of epiphytic algal communities revealed the presence of a novel species of

cyanobacteria at all confirmed AVM sites (Wilde et al., 2005). This species, a previously undescribed member of the order Stigonematales, was the dominant epiphyte during the AVM season in those water bodies where AVM has been observed most frequently and was the dominant epiphyte associated with hydrilla that induced AVM in laboratory mallards (Birrenkott et al., 2004). One hypothesis is that the etiologic agent of AVM is a neurotoxin produced by this cyanobacterium. The purpose of this study was to further examine the relationship between the Stigonematalan species and AVM induction, using animal feeding trials. In two separate trials, we attempted to induce AVM in animal models by feeding aquatic vegetation containing large quantities of the Stigonematalan species. Control animals received vegetation of the same type that did not contain the Stigonematalan species. In addition, we attempted to examine the time period necessary to induce AVM in the laboratory and were able to further explore the temporality of AVM occurrence.

Materials and Methods

Study Area

Aquatic vegetation (*Hydrilla verticillata* and associated materials) was collected from three sites in South Carolina (SC). Hydrilla containing the Stigonematalan species was collected from J. Strom Thurmond Lake (JSTL) (34 40’40’’ N, 82 13’0’’ W) and Davis Pond (33 42’23”N, 82 08’15”W). JSTL is a 28,700 ha reservoir located on the Savannah River on the border of South Carolina and Georgia and is a site of previous AVM epornitics. For this study,

hydrilla was collected from the Parksville Recreation Area, located along the southeast region of the lake. The Stigonematalan species was the most prevalent epiphyte associated with the hydrilla. Davis Pond is a 1.6 ha private pond located 6.8 km east of JSTL. The pond had been colonized by hydrilla during the summer of 2003 and no AVM morbidity or mortality had been observed previously at this site. At the time of the study, the Stigonematalan species was determined to be the most prevalent epiphytic species.

Control hydrilla was collected from Lake Marion, SC (33 32'18"N, 80 16'05"W), a 44,500 ha reservoir with no known AVM morbidity or mortality. Hydrilla was collected from the Potato Creek Embayment area. This site was chosen because it contained an abundance of hydrilla and associated epiphytes, but contained no observable colonies of the suspect Stigonematalan cyanobacteria. American coots were also collected from this site and analyzed for AVM lesions to ensure that the disease was not present.

Trial I

Mallard ducks and domestic chickens served as animal models. Sixteen adult male mallards were purchased from Whistling Wings (Hanover, Illinois, USA) and sixteen adult female chickens were obtained from the Clemson University Morgan Poultry Center (Clemson, South Carolina, USA). All birds were obtained 14 October 2003 and were housed in Godley-Snell Research Center at Clemson University. Both species were randomly divided into two experimental groups, treatment and control, with eight birds in each group.

Animals were housed according to experimental group. Treatment birds were fed hydrilla from JSTL and control birds were fed hydrilla from Lake Marion.

Hydrilla was collected from Lake Marion 14 October 2003 and from JSTL 7 October 2003. Hydrilla was collected with a rake from depths of up to 1.5 m and transported in covered plastic containers. A sub-sample from each site was examined microscopically to determine the associated epiphytes. Three random entire leaves were mounted on a glass slide and the average surface area covered by the cyanobacterial epiphytes was measured using epifluorescence microscopy. Surface area coverage was classified into four categories: present (1-25%), common (26-50%), abundant (51-75%), or dominant (76-100%). Real-time PCR was also performed to genetically confirm the presence and abundance of the Stigonematalan species (Williams et al., 2007), as described below. All collected vegetation was frozen at -20°C. Half of the hydrilla was distributed into gallon-size plastic bags before freezing for use in the feeding trials and the remaining half was stored at -20°C for potential future toxin analysis. Administration of hydrilla began 15 October 2003. All hydrilla was thawed before feeding. For mallards, hydrilla was given twice daily and placed in swim tanks for birds to consume *ad libitum*. Each group of mallards (n=8) received approximately 1000 - 1700 g wet weight (ww), although this measurement includes a substantial amount of water so the actual mass of hydrilla would be much less. Chickens received hydrilla twice daily as well, placed in feeding trays. Each group of chickens (n=8) received approximately 500-1200 g ww daily. All birds were given progressively less hydrilla as the number of birds per experimental group

was reduced each week, as described below. Both species received supplemental commercial feed for one hour twice daily and water was available *ad libitum*. Behavior was observed twice daily and weights were recorded twice weekly.

Following the first week of feeding, one-fourth of the birds (two treatment and two control of both species) were sacrificed each week, with the last group of birds receiving hydrilla for four weeks. Birds were euthanized by CO_2 and whole brains were removed. Brains were placed in 10% neutral buffered formalin and transported to the Southeastern Cooperative Wildlife Disease Study (SCWDS, Athens, Georgia, USA) for analysis.

Trial II

Trial II was conducted 18 November – 17 December 2003. Eighteen adult male mallard ducks were received from Whistling Wings 18 November. Mallards were housed at Godley-Snell Research Center according to experimental group.

The study sites for this trial included JSTL, Davis Pond, and Lake Marion. Hydrilla was collected weekly at each site during the course of the study, beginning 18 November. Sub-samples of hydrilla were examined for epiphytes as in Trial I. Quantitative real-time PCR was performed on select samples. Collection methods were identical to Trial I, with the exception that half of the hydrilla used in the feeding trials was kept at room temperature while the other half was frozen, and subsequently thawed, prior to feeding. This was done to determine if freezing the hydrilla had an impact on viability of the etiologic agent. Therefore, birds were randomly divided into six experimental groups, with three

birds per group: 1) JSTL, frozen hydrilla; 2) JSTL, fresh hydrilla (kept at room temperature); 3) Davis Pond, frozen hydrilla; 4) Davis Pond, fresh hydrilla; 5) Lake Marion, frozen hydrilla; and 6) Lake Marion, fresh hydrilla. Feeding methods were the same as in Trial I, with birds receiving hydrilla twice daily. Each group of birds (n=3) received approximately 500 to 1200 g ww daily. Behavior was observed twice daily and weights were recorded twice weekly. Due to the smaller experimental groups (n=3) in Trial II, all birds were sacrificed after four weeks of feeding. However, two birds were euthanized prior to this date after developing signs of neurological impairment. Methods of euthanasia and brain analysis were the same as in Trial I.

Real-time PCR Analysis

Real-time PCR analysis was conducted using methods developed by Williams et al. (2007). From each environmental location tested, 0.5 g of hydrilla leaves were weighed. DNA for real-time PCR was extracted using the UltraClean™ Plant DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, California, USA) following the kit protocol. All real-time PCR assays were conducted using the SmartCyler® System (Cepheid, Sunnyvale, California, USA). Reaction protocols for each assay consisted of three stages: two denaturation stages at 96°C for 75 s, 45 cycles at 96°C for 5 s, and an annealing stage at 58°C for 40 s. Reaction mixtures from each assay consisted of two flanking primers and an internal fluorescent-labeled probe developed by Williams et al. (2007) (Stig 1069 forward primer, primer sequence

5'-AGGGTGGGCACTCTAAAGA-3'; Stig 1248 reverse primer, primer sequence 5'-CAGCCTTCGATCTGAATTG-3'; Stig 1209 probe, sequence 5'[6~FAM]CAAATCTCGTAAACCGTTGCTAATT[TAMRA~6~FAM]3'). The following reagents were added to each 25 µl reaction mixture: 0.2 µm primers and internal probe; 1 x PCR buffer (Promega, Madison, Wisconsin, USA), 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture (Stratagene, La Jolla, California, USA), 10 µM bovine serum albumin, and 0.2 U µl-1 *Taq* polymerase (Promega, Madison, Wisconsin, USA).

Results

Trial I

In Trial I, no birds developed any clinical signs of AVM and all brains were negative for AVM lesions. Mallard body weights remained relatively constant over the course of the study, with an average increase of 6.1% (± 8.0% [SD]) and no individual weight loss exceeding 3.0%. Mallards readily ate the vegetation, consuming almost all of the offered material. The chickens consumed much less material than the ducks, only consuming one-third to one-half of the material offered and had an average weight loss of 5.4% (± 5.6%) from the beginning of the trial to euthanasia. No individual weight loss exceeded 20%.

Cyanobacterial epiphytes associated with the vegetation collected for Trial I, and their relative abundance, are shown in Tables 1 and 2. Several genera were present on both Lake Marion and JSTL material, with the Stigonematalan species dominant on JSTL vegetation and absent from Lake Marion vegetation. Real-

time PCR confirmed the absence of Stigonematalan colonies on Lake Marion material, and reported a value of 577.96 cells/ml for JSTL material.

Trial II

In Trial II, two birds developed signs of neurological impairment consistent with AVM. On the afternoon of 23 November, five days after initiation of the study, a member of the JSTL Fresh group was observed leaning backwards with a slightly unsteady gait. The bird was still eating and drinking readily. On the morning of 24 November, the bird displayed more severe ataxia, including falling onto its side and backwards, and inability to walk properly. At this time, the bird was euthanized and the brain was placed in formalin.

On 1 December, thirteen days after initiation of the study, another member of the JSTL Fresh group developed clinical signs consistent with AVM. This bird was observed tilting forward and generally appeared off balance and lethargic. The bird was euthanized and the brain was stored in formalin.

Table 1. Percent leaf surface area coverage of cyanobacterial epiphytes on collected vegetation (*Hydrilla verticillata*). Coverage was based on microscopic analysis of three random entire leaves and classified as either present (1-25%), common (26-50%), abundant (51-75%), or dominant (76-100%).

	Trial I[a]		Trial II[b]		
Cyanobacterial Genus	Lake Marion	JSTL[c]	Lake Marion	JSTL	Davis Pond
Anabaena	Present	Present		Present	Present
Aphanothece	Present	Present	Present	Present	
Gloeotrichia	Abundant		Common		
Gomphosphaeria			Present	Present	
Leptolyngbya	Common		Common	Present	Present
Microcoleus			Present		
Oscillatoria				Present	Present
Phormidium			Present		
Pseudanabaena		Present	Present		Present
Spirulina			Present		
Stigonematales sp.		**Abundant**		**Dominant**	**Abundant**
Trichormus	Common	Present	Common		Present

[a]Vegetation collected 7 October 2003 for JSTL, 14 October 2003 for Lake Marion.

[b]Vegetation collected 18 November 2003. See Table 2 for Stigonematales coverage on additional collection dates.

[c]J. Strom Thurmond Lake

Table 2. Percent leaf surface area coverage of the Stigonematales species on collected vegetation (*Hydrilla verticillata*). Coverage was based on microscopic analysis of three random entire leaves and classified as either present (1-25%), common (26-50%), abundant (51-75%), dominant (76-100%), or ND (not detected). Quantitative real-time PCR performed on select samples is listed in parentheses below coverage.

	Lake Marion	JSTL[a]	Davis Pond
Trial I			
7 October	----------[b]	Abundant (577.96 cells/ml)	----------
14 October	ND	----------	----------
Trial II			
18 November	ND	Dominant	Abundant (162.59 cells/ml)
25 November	ND	Abundant	Abundant
2 December	ND	Abundant (345.46 cells/ml)	Common
9 December	ND	Abundant	Common

[a]J. Strom Thurmond Lake

[b]No collection at this site

All other birds in Trial II remained clinically normal during the study. All weights remained relatively constant, with an average weight gain of 6.8% (± 11.5%) over the course of the study and no individual weight loss greater than 8.0%. The birds readily consumed the majority of the vegetation offered. Microscopic examination of brain tissue revealed the presence of AVM lesions in ten mallards, including the two displaying clinical signs. All six of the birds receiving JSTL hydrilla developed AVM, and four of the six birds receiving hydrilla from Davis Pond developed the disease (two out of three birds in both the Davis Fresh and Davis Frozen groups). No AVM lesions were observed in any bird receiving hydrilla from Lake Marion.

Cyanobacterial epiphytes associated with vegetation collected for Trial II are shown in Tables 1 and 2. The Stigonematalan species was not observed on Lake Marion material, though several other cyanobacterial genera were present. Leaf coverage of the Stigonematalan species on JSTL hydrilla was classified as abundant to dominant (51-100% leaf coverage), and quantitative real-time PCR on the 2 December 2003 collection reported a value of 345.46 cells/ml. Leaf coverage for Davis Pond was classified as common to abundant (26 -75% leaf coverage), and quantitative real-time PCR on the 18 November 2003 collection reported a value of 162.59 cell/ml.

Discussion

No birds in Trial I contracted AVM, apparently due to the absence or insufficient concentration of the causative agent in the collected vegetation. At

the time there was concern about whether or not freezing the material would affect the viability of the AVM agent, but results from Trial II have demonstrated that freezing did not have an effect, which is consistent with previous studies (Fischer et al., 2003; Lewis-Weis et al., 2004). Despite being housed in larger groups, ducks in Trial I had access to approximately the same amount of hydrilla as those in Trial II, and all birds appeared to eat readily. The chickens however, consumed much less hydrilla than the mallards and were therefore not used in Trial II. Chickens have been shown to be susceptible to AVM and an acceptable animal model for vegetation feeding trials in another study (Lewis-Weis et al., 2004).

Ten of twelve treatment ducks in Trial II were diagnosed with AVM lesions, indicating that at least some vegetative samples from both JSTL and Davis Pond collected during November and December contained the causative agent in sufficient quantity to produce lesions. The hydrilla collected from JSTL during the week of November 18 certainly contained the etiologic agent, as the bird that displayed clinical signs at five days had only consumed material from this collection.

Hydrilla was collected from the same site at JSTL for both trials; however, only that material collected during the second trial, collected November 18 - December 9, induced AVM, while material collected in October did not. The lack of AVM induction with earlier collections is consistent with other observations of AVM seasonality. Previous studies have reported the occurrence of AVM from November through March or April, with the highest prevalence in November and

December (Rocke et al., 2002; Fischer et al., 2002). On JSTL specifically, the majority of diagnostic accessions submitted to SCWDS since 1998 were found November – January, although active surveillance of coots on JSTL from 1998-2004 revealed the presence of lesions from October to March (Fischer et al., 2006). At the time of our collections in 2003, active surveillance of coots revealed no prevalence of lesions in October, but lesions were present in 22% and 7% of coots collected in November and December, respectively (Fischer et al., 2006). This marked seasonality supports the idea that AVM is the result of a naturally produced toxin.

Factors that initiate toxin production in cyanobacteria and other algal species are not precisely known (Haider et al., 2003). Toxins are usually associated with bloom formation, which can occur in response to environmental factors such as a change in nutrient concentrations, temperature, or light. In AVM reservoirs, the suspect Stigonematalan species experiences a bloom beginning late summer to early fall, occurring in abundance until the vegetation senesces during the winter months and it loses a suitable substrate (S. B. Wilde, unpubl. data). Observations of cyanobacterial epiphytes and PCR confirmed the presence of the Stigonematalan species during both the October and November collections. Comparable quantities were present for all, except for higher leaf coverage (76-100%) for the November 18 JSTL collection than the remaining November collections and October collection (51-75%).

The fact that the October material did not produce AVM does not imply that the Stigonematalan species is not involved, only that it may not have been

actively producing toxin at the time. While changes in algal toxin concentrations can often be explained by changes in algal abundance, there are many instances where there is no correlation between numbers of cyanobacteria and toxin production (Kardinaal and Visser, 2005). The same environmental factors that influence bloom formation – light, temperature, pH, nutrient ratios and concentrations – can also influence toxin dynamics. In addition, toxic and non-toxic strains of cyanobacteria can occur within the same species, further complicating the relationship between cyanobacterial biomass and toxin presence and concentration (Kardinaal and Visser, 2005).

It was our intention in Trial I to study the time of lesion formation by feeding our test animals vegetation from a single collection over a one month time period and euthanizing them at different time points, but no birds developed visible AVM lesions. However, clinical signs of AVM were observed in one bird five days after initial exposure to the hydrilla in Trial II. This quick time of onset has been observed previously with sentinel birds released onto an AVM affected reservoir (Rocke et al., 2002). It is unclear when the other birds that contracted AVM in the present study may have developed lesions.

Of the ten birds in the second feeding trial to have confirmed lesions consistent with AVM, two were observed with clinical signs of the disease. This frequency is consistent with the previous laboratory trial that successfully reproduced AVM in mallards, in which one of six ducks with confirmed lesions displayed clinical signs (Birrenkott et al., 2004). It is not clear why some birds display clinical signs and others do not. It may be related to the amount of the

etiologic agent that the bird ingests, the duration of exposure, variation in individual susceptibility and health of the bird, or some other factor. Lewis-Weis et al. (2004) reported a higher prevalence of clinical signs in chickens affected with rickets than in birds of good nutritional status. The mallards developing clinical signs in this study did not have any obvious signs of impaired health compared to the other birds, but could have had undetected conditions making them more susceptible. Thus far there appears to be no correlation between presence of clinical signs and severity of brain lesions. Birds with severe lesions can appear clinically normal while those with signs of neurological impairment can have mild to moderate lesions (Rocke et al., 2002; Fischer et al., 2003). Regardless of the reason, it is clear that many birds that develop AVM lesions do not develop clinical signs. This suggests that the disease may go undiagnosed unless active surveillance is conducted.

While not conclusive, this study provides further evidence that AVM induction may be linked to a newly discovered cyanobacterial species. At the least, this novel Stigonematalan species appears to be a good indicator of AVM disease potential, as material collected from two separate study sites based on the presence of this species induced AVM. We were able to correctly predict the presence of the AVM causative agent in Davis Pond due to the existence of two factors: the abundant submerged aquatic vegetation and the associated abundant Stigonematalan cyanobacteria. However, since the control and treatment hydrilla were collected from different study sites, factors other than presence or absence of the Stigonematalan species may be involved and further study is needed.

Vegetation collected from the same reservoir, some containing the Stigonematalan species and some without, would provide more convincing evidence. However, no reservoirs provided such a situation at the time of our collections. In addition, due to the nature of the epiphytic growth of cyanobacteria on the vegetative material, it is impractical, and perhaps not possible, to separate the cyanobacterial colonies from the vegetation in order to test it separately.

The Stigonematalan species is currently being cultured in the laboratory and future experiments include the testing of this material by mallard bioassay. In addition, experiments are underway involving attempts to extract the putative AVM toxin from collected vegetation samples. Much research is still needed to determine the cause of AVM, as well as to provide more information on the pathology, range of susceptible species, implications for wildlife populations, and management strategies.

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CHAPTER 2

AN EXTRACT OF *HYDRILLA VERTICILLATA* AND ASSOCIATED EPIPHYTES INDUCES AVIAN VACUOLAR MYELINOPATHY IN LABORATORY MALLARDS

Introduction

Avian vacuolar myelinopathy (AVM) is a neurological disease affecting bald eagles (*Haliaeetus leucocephalus*), American coots (*Fulica americana*), and other birds in the southeastern U.S. First observed in 1994, AVM is responsible for the deaths of over 100 bald eagles and estimates of over a thousand coots (Wilde et al., 2005).

AVM is characterized and diagnosed by a spongy degeneration of the white matter of the central nervous system, specifically an intramyelinic edema (Thomas et al., 1998). Affected birds may display clinical signs of neurological impairment, including difficulty in flying, swimming, and/or walking. However, not all birds with AVM lesions display clinical signs, thus pathological evaluation of brain tissue is necessary for diagnosis. The pathology of AVM is consistent with several known chemicals, including triethyltin, hexachlorophene, and bromethalin (van Gemert and Killeen, 1998), but no significant concentrations of these or other known toxins have been detected in tissues of affected birds or environmental samples (Thomas et al., 1998; Dodder et al., 2003). There has also been no evidence linking AVM to infectious agents and the disease was not found

to be contagious (Thomas et al., 1998; Larsen et al., 2003). Although the etiology of the disease remains unknown, recent investigations have suggested that the causative agent for AVM is a cyanobacterial toxin (Birrenkott et al., 2004; Wilde et al., 2005; Wiley, 2007, Chapter 1).

Previous investigations have established that AVM is site-specific and seasonal (Rocke et al., 2002), associated with a number of southeastern reservoirs with epizootics occurring during late fall to early winter. Coots and other waterfowl contract the disease by feeding on aquatic vegetation (Birrenkott et al., 2004; Lewis-Weis et al., 2004; Rocke et al., 2005), and bald eagles and other avian predators contract the disease by consuming affected prey (Fischer et al., 2003). All AVM-affected reservoirs contain extensive exotic aquatic macrophytes, but the same or similar macrophytes are also abundantly present in other nearby aquatic systems where AVM is not observed. Therefore, it has been hypothesized that the causative agent is something associated with the macrophytes, rather than the macrophytes themselves (Birrenkott et al., 2004; Lewis-Weis et al., 2004).

The macrophyte hydrilla (*Hydrilla verticillata*), that induced AVM in a laboratory controlled, mallard (*Anas platyrhynchos*) feeding study (Birrenkott et al., 2004), was shown to contain large quantities of an epiphytic cyanobacterial species. Colonies of this species, a previously uncharacterized member of the order Stigonematales (Williams et al., 2007), covered 50-90% of the leaf surface of the hydrilla. Surveys of all known AVM reservoirs, and similar reservoirs where AVM does not occur, revealed a strong positive correlation between the

presence of this cyanobacterial species and AVM (Wilde et al., 2005). Researchers have thus postulated that this cyanobacterial species may be producing a toxin that causes AVM in birds such as eagles and coots.

Cyanobacteria are ubiquitous in fresh, marine, and brackish waters, as well as terrestrial environments all over the globe. Numerous genera are known to produce toxins, which can have hepatotoxic, neurotoxic, or dermatotoxic effects in human and wildlife populations (Codd, 1995). The current list of known toxins is incomplete, as new and emerging toxins are recognized due to advances in analytical techniques and an increasing awareness of the potential for cyanotoxin poisoning events (Codd, 1995; Codd et al., 2001; Cox et al., 2005).

To date investigations on AVM have been limited to feeding studies utilizing whole tissues or vegetation samples without any quantitative indication of the presence of the AVM toxin. The aim of the present study was therefore to develop a method to extract the causative agent of AVM from aquatic vegetation as the first step towards purification and characterization of the AVM toxin. Successful isolation of a partially purified toxic extract will provide an invaluable tool for *in vitro* and *in vivo* toxicology studies including investigations determining the mechanism of action.

Materials and Methods

Vegetation Collection

Hydrilla was collected from J Strom Thurmond Lake (JSTL), South Carolina, USA, a site of previous and current AVM epizootics (Fischer et al.,

2002; Fischer et al., 2003; Lewis-Weis et al., 2004). This vegetation contained abundant quantities of an epiphytic cyanobacterium that has been suggested as a possible source of the disease agent (Wilde et al., 2005), with leaf surface area coverage ranging from 51-100%. Control hydrilla was collected from Lake Marion, South Carolina, USA, a reservoir with no known history of AVM occurrence. The suspect cyanobacterium was absent in all collections made from Lake Marion, as confirmed by microscopic analysis of representative leaves.

Samples were collected weekly, for four weeks, from mid-November to mid-December 2003. Collections were made with a rake, from the surface to a depth of approximately 1.5 m, and samples were stored and transported in covered plastic containers (200 L, Rubbermaid, Atlanta, Georgia, USA). Excess water was drained and samples were stored at -20°C, except as described below for the mallard vegetation bioassay.

Mallard Bioassay I - Vegetation

To confirm the presence or absence of the AVM causative agent, half of the vegetation collected was fed *ad libitum* to farm-reared mallard ducks in the laboratory. Detailed results of this study are reported in Chapter 1 (Wiley, 2007). Briefly, six ducks were fed vegetation from JSTL and six ducks fed vegetation from Lake Marion. Three ducks in each group received fresh vegetation that had not been frozen, but kept at room temperature. The remaining three ducks received vegetation that had been frozen at -20°C, and thawed prior to feeding. All six ducks receiving JSTL vegetation developed white matter lesions consistent

with AVM, confirming the presence of the AVM agent. No ducks receiving Lake Marion vegetation developed AVM lesions, confirming the absence of the AVM agent. This trial also confirmed that freezing does not affect stability of the AVM causative agent.

Toxin Extraction

The remaining half of the collected vegetation was used for a solvent extraction procedure. Frozen vegetation was lyophilized (Virtis Company, Gardener, New York, USA) and blended (Waring Commercial Blender, Waring, Torrington, Connecticut, USA) at medium speed for 1 min into a coarse powder in preparation for extraction. Each of the four weekly samples of vegetation was lyophilized and extracted separately, and dry weight of each sample was recorded prior to extraction. Dry weights of weekly samples ranged from 418.48 – 1219.88 g, with an average of 837.83 g. Dried, powdered vegetation was loaded evenly into three to four, 2 L separatory funnels (200-300 g per funnel) and sequentially extracted with hexanes, acetone and methanol (Fisher Chemical, HPLC grade, Fisher Scientific, Hampton, New Hampshire, USA). Solvent was added to the vegetation until saturated (1-1.5 L of solvent per funnel) and the sample was mechanically extracted by shaking for five, 2 min periods over 1 h. The extract was then allowed to drain, and the sample was rinsed with a final volume of solvent (0.5 L). Drained solvents were pooled. This process was then repeated with the next solvent. The collected extracts were filtered through 70 mm Whatman qualitative grade filter paper (Whatman, Inc., Florham Park, New

Jersey, USA) to remove particulates, and solvent was removed by a rotary evaporator (Buchi Rotavapor, Buchi, Flawil, Switzerland) with a water bath set no higher than 28°C. When dry, the extracts were re-suspended in the appropriate volume of solvent to a concentration of 10 g vegetation/ml solvent, based on the initial dry weight of the vegetation. Ten percent of this volume was archived for future *in vitro* experiments, and the remaining 90% was transferred to a glass storage jar for use in the *in vivo* mallard extract bioassay. All extracts were stored at -20°C.

Mallard Bioassay II - Extracts

In order to determine whether any extract(s) contained the AVM causative agent, they were evaluated by a mallard bioassay. Eighteen adult male mallards (average body weight: 1017 g) were obtained from Frost Waterfowl Trust (Georgetown, South Carolina, USA) and housed in separate floor pens by experimental group at Godley-Snell Research Center, Clemson University. Birds were weighed upon arrival and randomly assigned to one of six experimental groups (three birds per group): JSTL hexanes extract, JSTL acetone extract, JSTL methanol extract, Lake Marion hexanes extract, Lake Marion acetone extract, and Lake Marion methanol extract. Commercial feed and water were provided *ad libitum*, except as described for gavage. Birds were allowed to acclimate for three days prior to initiation of treatment.

Prior to dosing, extracts were dried under a N_2 stream and re-suspended in non-toxic vehicles. Hexanes extracts were re-suspended in corn oil (MP

Biomedicals, Irvine, California, USA), acetone extracts were re-suspended in a 50:40:10 (v:v:v) mixture of corn oil: deionized water: propylene glycol (EMD Chemicals, Gibbstown, New Jersey, USA), and methanol extracts were re-suspended in a 90:10 (v:v) mixture of deionized water: propylene glycol. Each weekly vegetative extract was re-suspended and dosed separately over the course of 1 week (4 weeks in total), so as to mimic the conditions in the mallard vegetation bioassay. This was done to avoid a possible dilution of the toxin if weekly collections had contained differing toxin concentrations. Extracts were re-suspended in the appropriate volume of vehicle to deliver the entire extract in three equivalent doses over one week. Week one extracts were re-suspended in 45 ml of vehicle, to provide a 5 ml dose volume per duck/per dosing. Dose volume was reduced to 4 ml following the first week in an attempt to reduce regurgitation that occurred with some birds, as explained in the results section. Therefore, the remaining extracts were re-suspended in 36 ml, to provide a 4 ml dose volume per bird/per dosing.

Mallards were orally gavaged with the re-suspended extracts three times a week for four weeks. Administration of the extracts was immediately followed by a rinse of the respective vehicle to help wash residual extract out of the feeding tube. Initial rinse volumes were 3 ml, but were reduced to 2 ml following the first week of dosing due to the observed regurgitation. Therefore, total dose volume including extract and rinse was 8 ml during the first week and 6 ml for the remainder of the study. Food was removed approximately 4 h prior to treatment

and provided immediately post-treatment. Birds were observed for at least 30 min post-treatment and any regurgitation was noted.

All birds were observed twice daily during the duration of the study and weights were recorded twice weekly. Birds were euthanized three days following the final dose administration, 28 d after the treatment was initiated. Birds were sacrificed by CO_2 asphyxiation with a Euthanex CO_2/O_2 blending system (Euthanex E-23000, Euthanex Corporation, Palmer, Pennsylvania, USA), using a 70:30 CO_2:O_2 mixture until birds lost consciousness, followed by 100% CO_2 to complete euthanasia. Whole brains were removed and placed in 10% neutral buffered formalin in preparation for AVM diagnosis. Whole blood and other tissues, including liver, kidney, pectoral muscle, gizzard, intestines, and adipose tissue were also collected and archived at -20°C. Brain analysis was conducted at the Southeastern Cooperative Wildlife Disease Study by methods previously described (Fischer et al., 2003).

Results

No clinical signs of neurological disease, including ataxia, incoordination, or lethargy, were observed during the trial. All animals maintained a fairly constant body weight throughout the study. On day 15 of the treatment period, a bird in the AVM-positive methanol group broke a wing during removal from the pen for treatment. This bird was immediately euthanized and necropsied.

Following gavage, several birds were observed regurgitating the administered dose, usually within five minutes of administration. This occurred

in the JSTL and Lake Marion methanol groups following the first two treatments, with at least one bird in both groups regurgitating. Since the birds were housed in groups, we were unable to determine with confidence how many birds regurgitated or which bird regurgitated unless it was observed directly. In an attempt to prevent regurgitation, the total dose volume was reduced to 6 mL, as previously described. Following the next two dose administrations, at least one bird in both methanol groups again regurgitated. However, for all successive treatments, no regurgitation was observed in the Lake Marion methanol group. At least one bird, and often all birds, in the JSTL methanol group continued to regurgitate after each treatment. In addition, at least one bird in the JSTL acetone group regurgitated three times during the study, and at least one bird in the JSTL hexane and Lake Marion acetone groups regurgitated once. The occurrences of regurgitation by experimental group are shown in Table 3. There was a significant difference in the occurrence of regurgitation between the JSTL methanol group and all other groups ($p<0.001$, Fisher's exact test).

The results of the brain analysis are shown in Table 4. All three birds in the JSTL methanol group developed definitive lesions of moderate severity consistent with AVM (Figure 1). One bird in the JSTL acetone group developed possible mild lesions (Figure 2). All other birds had no apparent lesions (Figure 1). Brain slides are archived at the Marine Resources Research Institute, South Carolina Department of Natural Resources, Charleston, South Carolina, USA.

Table 3. Occurrence of regurgitation in mallards after administration of an extract of *Hydrilla verticillata* and associated epiphytes collected from Lake Marion, South Carolina or J. Strom Thurmond Lake, South Carolina.

	Lake Marion		J. Strom Thurmond Lake	
Extract	Doses n	Doses Regurgitated[a] n	Doses n	Doses Regurgitated[a] n
Hexanes	12	0	12	1
Acetone	12	1	12	3
Methanol	12	4	12	12

[a]Indicates the number of times at least one bird in group regurgitated.

Table 4. Presence of AVM-type brain lesions in mallards after administration of an extract of *Hydrilla verticillata* and associated epiphytes collected from Lake Marion, South Carolina or J. Strom Thurmond Lake, South Carolina.

	Lake Marion		J. Strom Thurmond Lake	
Extract	n	Birds with lesions	n	Birds with lesions
Hexanes	3	0	3	0
Acetone	3	0	3	1[a]
Methanol	3	0	3	3

[a]Possible mild lesions. Results inconclusive.

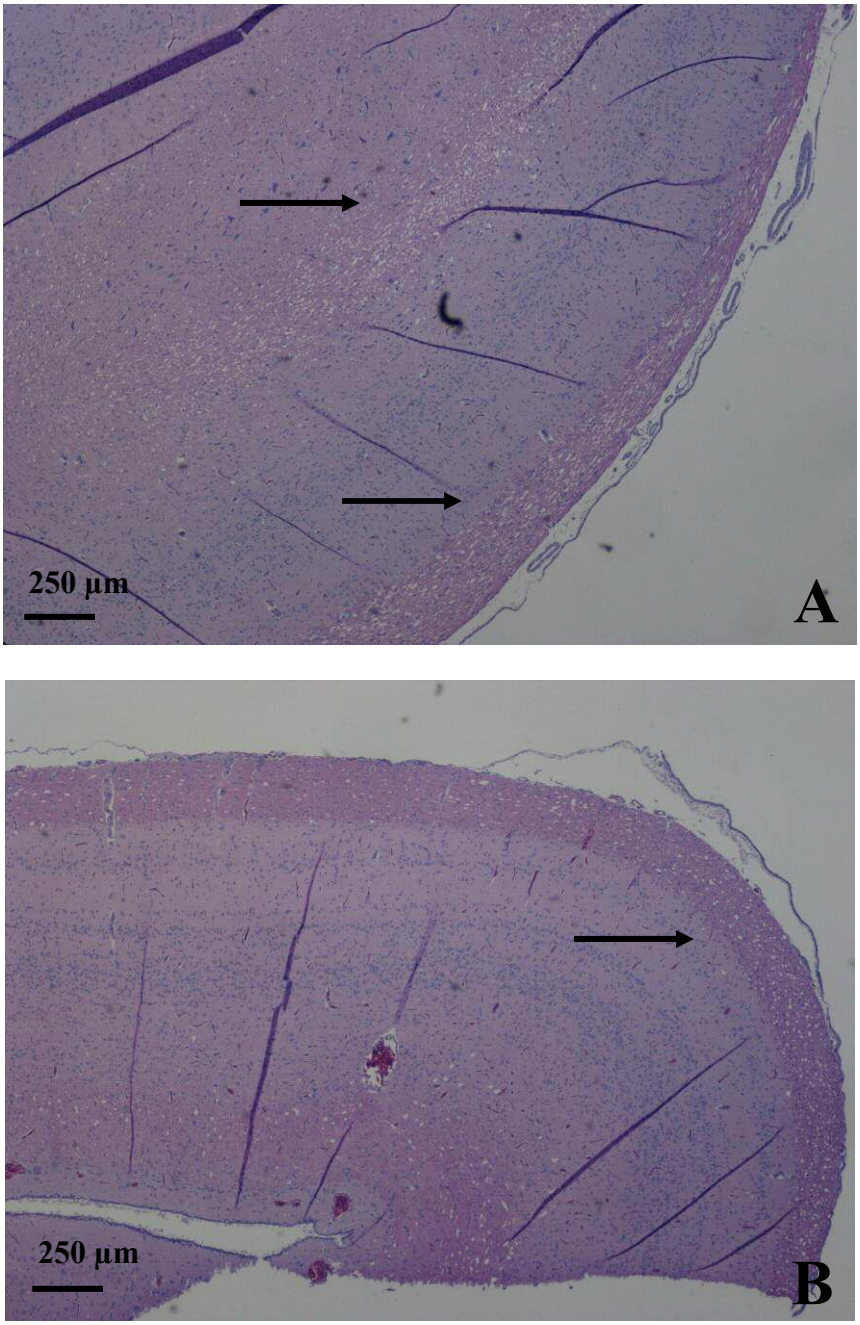

Figure 1. Light micrograph images of brain sections of mallards given J. Strom Thurmond Lake methanol extract (A, B, C) or Lake Marion methanol extract (D). A, B, and C have definitive lesions of moderate severity in the white matter tracts (arrows) and were diagnosed as having AVM. Brain D has no observable lesions and was diagnosed as not having AVM.

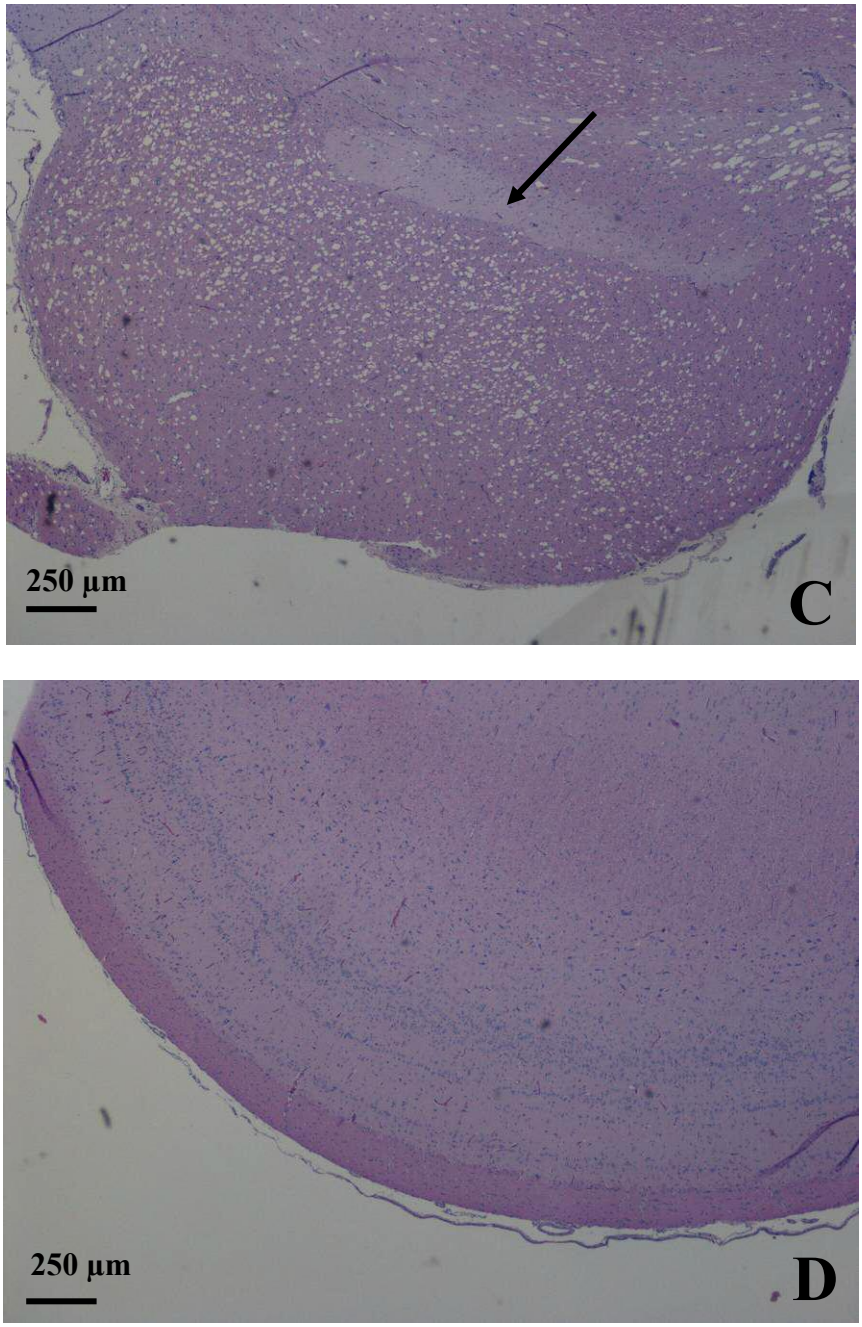

Figure 1. Light micrograph images of brain sections of mallards given J. Strom Thurmond Lake methanol extract (A, B, C) or Lake Marion methanol extract (D). A, B, and C have definitive lesions of moderate severity in the white matter tracts (arrows) and were diagnosed as having AVM. Brain D has no observable lesions and was diagnosed as not having AVM (Continued).

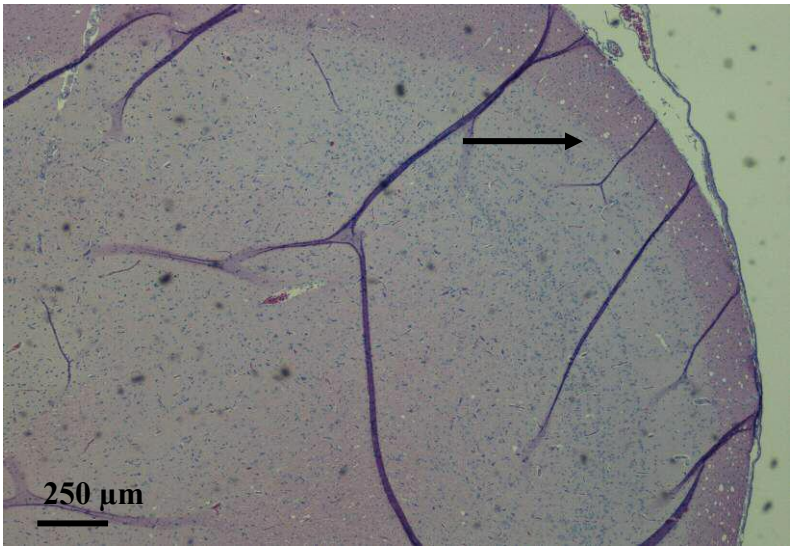

Figure 2. Light micrograph image of the optic lobe of a mallard gavaged with J. Strom Thurmond Lake acetone extract. Mild lesions are visible in the outer layer of white matter (arrow) but were not definitive. Therefore a diagnosis of AVM was not made.

Discussion

This study describes the successful extraction of the putative AVM toxin from hydrilla and its associated epiphytic cyanobacterial flora. Results of previous studies have ruled out many other hypothesized toxicants, including bacterial and viral agents, and the disease was not found to be contagious (Thomas et al., 1998; Larsen et al., 2003). The pathology of AVM was consistent with acute toxicosis, with lesions similar to those caused by known toxicants and no observed immune response (Thomas et al., 1998).

The toxin appears to be efficiently extracted with methanol, suggesting a polar compound. One bird administered the acetone extract had possible mild lesions, though the results were inconclusive. If this bird did indeed have AVM, it would not be surprising that toxin was also present in the acetone extract, as the polarity of acetone and methanol do not differ greatly (Snyder, 1978; Marcus, 1998; Fitzpatrick and Dean, 2002). Both solvents also have similar octanol/water partition coefficients (K_{ow} acetone = -0.24; methanol = -0.70) (Marcus, 1998). Methanol, however, has a much greater hydrogen bonding ability than acetone (Marcus, 1998; Fitzpatrick and Dean, 2002), another factor which can play a role in extraction efficiency. It is also important to remember the matrix effects that could be involved in the extraction process. While hexanes or acetone could have similar properties to the toxin, methanol may have been required to release the toxin from interactions within the matrix. Therefore, while it appears the toxin is polar, further fractionation of the crude extract will be necessary to provide a more accurate and detailed description of its physical properties.

The AVM toxin appears to be stable, surviving at -20°C for at least one year and at room temperature for at least several days. The highest temperature to which the extracts were exposed was 28°C. It is not known whether or not the toxin would be stable at higher temperatures. In addition, the toxin does not appear to be volatile, as it survived extensive lyophilization and vacuum concentration. However, since the toxin is not known there is no method available to quantify its concentration and we cannot gauge the efficiency of the extraction procedure or rates of degradation or loss.

No typical clinical signs of disease were observed in the AVM-positive mallards; however, previous field and laboratory data have shown that many pathologically affected birds do not display signs of disease (Rocke et al., 2002; Fischer et al., 2003; Birrenkott et al., 2004; Lewis-Weis et al., 2004; Rocke et al., 2005). It is not yet known why some birds develop clinical signs whereas others do not. The occurrence and severity of clinical signs has not been positively correlated to the severity of brain lesions. Toxin concentration, exposure time, route of administration, and individual susceptibility or health status of the bird may play a role. Birds with severe lesions can appear clinically normal while birds displaying signs of neurological impairment may have mild or moderate lesions (Rocke et al., 2002; Fischer et al., 2003). It is possible that the lesions are unrelated to clinical signs, and that a second, unidentified pathology exists.

The results of this study suggest that the extracted AVM toxin elicits an emetic response. Birds in the AVM methanol group that developed lesions consistently regurgitated following dose administration. While birds in several other dose groups, including controls, also regurgitated during the study, they did not do so consistently. Regurgitation as a method of rejection has not been noted previously in other AVM research; however, this is the first study to administer the putative toxin in a concentrated form. Previous studies that have been successful at reproducing AVM in the laboratory have used either AVM contaminated vegetation (i.e., *Hydrilla verticillata*) or AVM positive coot tissues administered *ad libitum* (Fischer et al., 2003; Birrenkott et al., 2004; Lewis-Weis et al., 2004; Rocke et al., 2005). Nonetheless, mallards are prone to a

regurgitation response during toxicity testing which may be caused by the test substance itself, the volume administered, the vehicle or dosing technique, or a combination of these factors (OECD, 2002). The mid-experiment reduction in dosing volume may have helped prevent regurgitation in the Lake Marion methanol group, but it had no effect on the frequency of regurgitation for the JSTL methanol birds. Many known toxins and toxicants are known to have an emetic effect, and it is possible that the AVM toxin is responsible for the increased frequency of regurgitation observed in the affected birds.

The time of disease onset for the affected birds is not known. At least one bird developed lesions within two weeks of treatment initiation, as the mallard euthanized due to a broken wing at day 15 of the treatment period was diagnosed with AVM. Previous studies have shown that clinical signs and lesions can develop as early as five days post-exposure to the causative agent (Rocke et al., 2002; Wiley, 2007, Chapter 1). It is not known whether or not disease onset occurs with a single acute exposure of a threshold concentration or can develop after chronic exposure to lower concentrations.

The results of the current study provide further evidence that the etiological agent of AVM is associated with aquatic vegetation such as hydrilla and is most likely not the vegetation itself, but rather something associated with the vegetation, given that the birds receiving Lake Marion control hydrilla extracts did not develop AVM lesions. Our working hypothesis is that AVM is caused by an algal or cyanobacterial toxin. The seasonal nature of AVM epornitics supports this hypothesis, as algal and cyanobacterial blooms are

successional with seasonal changes. Wilde et al. (2005) conducted algal surveys at all known sites of AVM epornitics, as well as additional control reservoirs, to look for algal or cyanobacterial species that were unique to AVM sites or of unusual abundance during the AVM season. A previously uncharacterized cyanobacterium of the order Stigonematales emerged as the prime suspect. This epiphytic cyanobacterium was associated with lake vegetation, particularly hydrilla, was observed consistently at all known AVM sites, and was the dominant epiphyte present at most AVM sites during the late fall to early winter, when AVM is commonly observed (Wilde et al., 2005). At sites where AVM birds are diagnosed most consistently, the Stigonematalan species covered up to 95% of the leaf surface area of the vegetation during these months. In addition, at control sites where AVM diagnosed birds are noticeably absent, the Stigonematales cyanobacterium was not dominant (<10% of the leaf surface area) and only infrequently observed.

The vegetation used in the present study was selected based on the recent AVM histories of each study site in addition to the presence or absence of the Stigonematalan species. JSTL hydrilla producing AVM was extensively covered by the Stigonematalan species, with 50-100% of the leaf surface area covered with colonies. In contrast, Lake Marion hydrilla, while containing numerous algal and cyanobacterial epiphytes, contained no Stigonematalan colonies, based on microscopic evaluation as well as genetic analysis using real-time PCR (Wiley, 2007, Chapter 1). While the most obvious differences in the JSTL and Lake Marion vegetation is the presence or absence of the Stigonematalan species, there

could be other unknown factors involved in disease onset. To confirm the link between the Stigonematalan cyanobacteria and AVM, the disease must be demonstrated from direct exposure of birds to pure cultures of the cyanobacteria species.

Many questions remain to be answered regarding AVM. In addition to determining the source of disease, research is needed concerning the pathology, range of susceptible species, and potential management strategies. Mechanism of action, exposure times and concentrations necessary to produce the disease, acute versus chronic exposure effects, and possible recovery and/or antidote strategies are all unknown. Although species susceptibility is currently confined to birds, the list of susceptible species continues to grow, where in particular, research focused on mammalian susceptibility is greatly needed. To date, no mammals have been positively diagnosed with AVM in the wild; however, there has been anecdotal evidence of possible clinical signs in a beaver (*Castor canadensis*) (Lewis-Weis et al., 2004). Several subsequent laboratory studies have investigated mammalian susceptibility in mice and swine, yet none of these animals developed lesions (Lewis-Weis et al., 2004; Rocke et al., 2005). The authors note, however, that the quantities consumed or duration of exposure may not have been adequate to induce disease.

The successful extraction of the toxin responsible for AVM will assist in answering many of the questions remaining about AVM, its etiologic agent, and its mode of action. In addition to being the first step toward isolating and characterizing the toxin, this successful extraction protocol for separation of the

AVM toxin from an environmental matrix (i.e., hydrilla – cyanobacterial complex) will result in numerous opportunities for further research. Researchers are currently limited to nonspecific, whole animal studies, for which large quantities of vegetation or tissue samples are required. The production of a toxic extract through an efficient solvent extraction protocol will improve the utility of *in vivo* studies and allow for testing of additional animal models such as mice that may not readily consume vegetation. In addition, the ability to extract the putative toxin will allow for *in vitro* testing. *In vitro* studies using cell and tissue cultures would provide essential data on the pathology and mechanism of action. Identification of an appropriate *in vitro* model will also provide a more rapid and sensitive toxin detection method than whole animal bioassays, which will be an essential tool for bioassay-guided fractionation for toxin purification and chemical characterization.

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CHAPTER 3

IN VITRO TOXICITY ASSOCIATED WITH THE ETIOLOGIC AGENT OF AVIAN VACUOLAR MYELINOPATHY

Introduction

Avian vacuolar myelinopathy (AVM) is a neurological disease affecting bald eagles (*Haliaeetus leucocephalus*), American coots (*Fulica americana*), and other avian species in the southeastern U.S. Since the first recorded AVM epizootic in 1994, over 100 bald eagles have died from the disease, and thousands of coots have been affected. The cause of AVM has yet to be determined, although it is associated with consumption of aquatic vegetation – or animals that have consumed aquatic vegetation – (Birrenkott et al., 2004; Lewis-Weis et al., 2004; Rocke et al., 2005) and has been linked to a novel cyanobacterial species in the order Stigonematales that grows on the vegetation (Wilde et al., 2005). The etiologic agent of AVM is thought to be a toxin, and it has been hypothesized that the epiphytic cyanobacterium is the source of that toxin.

AVM is characterized by a separation of the layers of the myelin sheaths in the central nervous system, a condition known as intramyelinic edema (Thomas et al., 1998). The brain tissue of AVM affected birds has a spongy appearance under light microscopy due to the vacuoles formed from the separated layers. Several compounds are known to cause this type of lesion, including triethyltin,

hexachlorophene, bromethalin, and others, but none of these compounds have been implicated in AVM.

The only current means of detecting and studying the AVM toxin is by *in vivo* animal studies. These studies have utilized several avian and mammalian species as animal models, typically last one to six weeks, and involve the administration of large quantities of vegetation, tissues, or other test materials. It is through these studies that researchers have been able to locate the source of exposure to the AVM toxin (aquatic vegetation and associated epiphytes), have demonstrated the conveyance of toxicity from prey items to predators (Fischer et al., 2003), and have explored mammalian susceptibility (Lewis-Weis et al., 2004).

Despite the utility of *in vivo* studies they have numerous disadvantages. The trials are labor intensive, generally require large quantities of test material, and take extensive time to plan and conduct. The endpoint of these studies – the determination of AVM characteristic lesions in the brain – also takes time to analyze. The purpose of this study was therefore to develop an *in vitro*, cell based bioassay for AVM toxin detection that could be used as an alternative to the large scale *in vivo* assays. In addition to reducing the need for animal subjects, an *in vitro* assay would allow for small-scale testing, using minimal amounts of the test solution/substance. The high throughput design and rapid analysis time of many of these assays allows for testing large numbers of experimental groups and replicates quickly and can therefore be used as an efficient guide to track toxicity in toxin fractionation experiments. *In vitro* assays also represent a simplified

system in which cellular mechanisms and damage may be more easily explored and detected to study toxin mechanisms of action.

We have been successful at extracting the causative agent of AVM from vegetative samples (Wiley, 2007, Chapter 2). Crude extracts were produced from vegetation collected at both AVM-positive and AVM-negative sites and examined for toxicity by a mallard (*Anas platyrhynchos*) bioassay, which subsequently identified the crude methanol extract as containing the active toxin. The ability to produce these extracts has now allowed for the possibility of studying the AVM toxin *in vitro*. The purpose of this study was to examine the *in vitro* toxicity of these crude methanol extracts in an attempt to discover an endpoint that could be used in identifying AVM toxin presence in a sample. Since the extracts were in a crude form, containing a large mixture of potentially bioactive compounds associated with the vegetation and the associated epiphytic communities, vegetative extracts from AVM-negative sites served as controls in all experiments.

Established cell lines served as the experimental model in all assays. Established, or immortal, cell lines are those that can replicate indefinitely in culture, being either derived from tumors or transformed to allow for indefinite division. They have several advantages over primary cultures, which are derived from animal tissues directly prior to testing and have a finite number of divisions. Established cultures are easily obtained from cell culture collections and easily maintained in the laboratory. These cell lines have yielded documented results and their strengths and weaknesses are well known. They allow for high

reproducibility between experiments and laboratories and are therefore a good system to use for *in vitro* assay development.

Materials and Methods

Cell Culture

To investigate the *in vitro* toxicity of the crude extracts, initial experiments examined the cytotoxic potential of the extracts to four established cell lines. Three mammalian cells lines were tested: two neuronal lines, PC-12 and Neuro-2a (N2a), and one glial line, C6. These cells are utilized routinely in the research community and a large body of literature exists on all. A quail neuroretinal cell line (QNR/D) was also included in the experiments as it was the only avian neuronal line available as an established culture. In addition to the cytotoxicity tests, extracts were further tested on C6 cells for the ability to induce apoptosis, cytoskeletal changes, and cell cycle disruption.

C6, PC-12, Neuro-2a (N2a) and QNR/D cells were obtained from the American Type Culture Collection (ATCC). C6 and PC-12 cells were maintained at 37°C with 5% CO_2 in Kaighn's modification of Ham's F12 medium with 2mM L-glutamine modified to contain 1.5 g/L sodium bicarbonate and supplemented with 15% horse serum and 2.5% fetal bovine serum. N2a cells were maintained at 37°C with 5% CO_2 in RPMI medium supplemented with 10% fetal bovine serum and 1.0 mM sodium pyruvate. QNR/D cells were maintained at 39°C with 5% CO_2 in Dulbecco's Modified Eagle's medium modified to contain 4.5 g/L glucose and 1.5 g/L sodium bicarbonate, supplemented with 10% fetal bovine

serum. C6 and N2a cells were routinely passaged twice weekly and QNR/D and PC-12 cells were passaged weekly. Cells in passages 5-30 were used in assays. All cells were grown in the absence of antibiotics.

Vegetation Extractions

All extracts were produced using methods developed in Chapter 2 (Wiley, 2007). Locations and dates of vegetation collections are shown in Table 5. AVM-positive extracts are defined as those collected at sites with a known history of AVM. The Stigonematalan cyanobacterium that has been linked to AVM was also present in these samples, as confirmed by microscopic examination (Williams, unpubl. data). AVM-negative extracts are defined as those collected from sites with no known history of AVM. Samples collected at these sites did not contain the Stigonematalan cyanobacteria, as confirmed by microscopic examination and PCR (Williams, unpubl. data). Frozen vegetation was lyophilized (Virtis Company, Gardener, New York, USA) and blended (Waring Commercial Blender, Waring, Torrington, Connecticut, USA) into a coarse powder in preparation for extraction and dry weight was recorded. Dried, powdered vegetation was then sequentially extracted with hexanes, acetone and methanol (Fisher Chemical, HPLC grade, Fisher Scientific, Hampton, New Hampshire, USA), filtered, and reduced by vacuum concentration. When dry, the extracts were re-suspended in the appropriate volume of solvent to a concentration of 10 g vegetation/ml solvent, based on the initial dry weight of the vegetation. Prior to cell testing, extracts were sterile filtered through 0.2 µm

Table 5. Date of collection and location for vegetation extracted for use in the *in vitro* experiments. "AVM-negative" refers to those sites which have no previous history of AVM. "AVM-positive" sites are those that have had documented AVM epizootics.

Sample ID	Collection Site	Collection Date
AVM-negative		
Potato Creek-A[a]	Potato Creek Embayment, Lake Marion, South Carolina	14 October 2003
Potato Creek-B[a]	Potato Creek Embayment, Lake Marion, South Carolina	17 November 2003
Dens Swamp	Dens Swamp Cove, Lake Marion, South Carolina	21 December 2005
SCWMA	Santee Cooper Wildlife Management Area, South Carolina	6 December 2006
Lake Istokpoga	Lake Istokpoga, Florida	12 December 2006
AVM-positive		
Parksville-A[ab]	Parksville Cove, J. Strom Thurmond Lake, South Carolina	18 November 2003
Parksville-B[ab]	Parksville Cove, J. Strom Thurmond Lake, South Carolina	25 November 2003
Petersburg[ab]	Petersburg Cove, J. Strom Thurmond Lake, Georgia	1 December 2005
Woodlake[b]	Woodlake, North Carolina	22 November 2005
DeGray	DeGray Lake, Arkansas	27 November 2006

[a]Samples validated by mallard bioassay.

[b]Samples were collected during documented AVM epizootics.

syringe filters (Waters Corporation, Milford, Massachusetts, USA). All extracts were stored at -20°C and crude methanol extracts were used in the assays.

Cytotoxicity Assays

MTT. Cytotoxicity was determined by measuring 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reduction, a measure of metabolic activity. Live cells (or metabolically active cells) are able to reduce MTT, normally a yellow color, into purple formazan crystals, which are solubilized and measured spectrophotometrically to indicate the number of viable cells present in relation to a control population. C6 cells were plated at 1 x 10^5/ml, N2a and PC-12 at 2 x 10^5/ml, and QNR/D at 3.5 x 10^5/ml in 96-well plates at 100 µl per well and allowed to attach overnight. Cells were dosed with crude methanol extract from Parksville-A (AVM-positive) and Potato Creek-B (AVM-negative), at 3.125, 6.25, 12.5, 25, 50, and 100 mg/ml. Control (no addition) and vehicle control (methanol only) groups were also included. All doses were added in 1 µl volumes (1% of total volume in well). Following exposure for 24 h, media was removed and replaced with new media containing 0.8 mg/ml MTT. Cells were further incubated 2 h at 37°C (C6, N2a, PC-12) or 3 h at 39°C (QNR/D). Following incubation, media was removed and 100 µl DMSO added to solubilize the MTT crystals. Absorbance was measured on a NOVOStar microplate reader (BMG Lab Technologies, Offenburg, Germany) at 544 nm. Background absorbance (from media only) was subtracted from all values. Triplicate wells from each treatment were averaged and expressed as percent of vehicle control.

Each extract was tested in 3-4 different culture preparations and average means from the independent tests were compared using a two-tailed, paired student's t-test as performed by MS Excel, with a p-value of < 0.05 considered significant. EC_{50} values were obtained following variable-slope concentration-response analysis using log-transformed concentration and normalized response as performed by GraphPad Prism software (v 5.00, GraphPad Software, Inc., San Diego, California, USA).

LDH. Cell death was also estimated in C6 cells by measuring the leakage of lactate dehydrogenase (LDH) into the culture medium as an indicator of plasma membrane damage. C6 cells were plated at 1 x 10^5/ml in 96-well plates at 100 µl per well and allowed to attach overnight. Prior to dosing, the medium was replaced with serum-free media, as serum contains LDH which can increase the background absorbance values in the assay. Cells were dosed with crude methanol extract from Parksville-A (AVM-positive) and Potato Creek-B (AVM-negative), at 3.125, 6.25, 12.5, 25, 50, and 100 mg/ml. Control (no addition), vehicle control (methanol only), and positive control (methanol plus 1% Triton X-100 to lyse cells) groups were also included. All doses were added in 1 µl volumes (1% of total volume in well). Following exposure for 24 h, the supernatant was collected, centrifuged at 5000 rpm for 10 min to remove cellular debris, and analyzed for the presence of LDH by the Takara LDH Cytotoxicity Detection Kit (Takara Bio Inc, Japan). This kit measures LDH activity by measuring the reduction of NAD to NADH/H by the LDH-catalyzed conversion of lactate to pyruvate. The reduced

NADH/H is then used in the reduction of the tetrazolium salt INT to formazan by diaphorase. The reduced formazan product, a red dye, was quantified by measuring the absorbance on a NOVOStar microplate reader at 485nm. Background absorbance (media only) was subtracted from all values and percent cytotoxicity was calculated as [(experimental value – vehicle control)/(positive control – vehicle control)] x 100. Triplicate wells from each treatment were averaged. The assay was performed three times with different culture preparations and average means from the independent tests were compared using a two-tailed, paired student's t-test as performed by MS Excel, with a p-value of < 0.05 considered significant. EC_{50} values were obtained following variable-slope concentration-response analysis using log-transformed concentration and normalized response as performed by GraphPad Prism software.

Apoptosis Assays

C6 cells were examined for apoptosis by two methods: (1) examining cell surface expression of phosphatidylserine (PS) and (2) measurement of caspase-3 activity. PS is a phospholipid normally associated with the cytoplasmic side of the cell membrane which is translocated to the outer leaflet of the membrane during apoptosis (Vermes et al., 1995). Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS, and when labeled with a fluorophore can be used to identify apoptotic cells by binding to exposed PS on the outer leaflet. Caspases are a family of proteases that mediate the events associated with apoptosis. Caspase-3 is an effector caspase that functions in cell

disassembly (Thornberry and Lazebnik, 1998) and its activity can be measured as an indicator of apoptotic cell death (Gurtu et al., 1997).

C6 cells were plated at ~5 x 10^5/ml in 6-well plates at 2 ml per well and allowed to attach overnight. Cells were exposed to crude methanol extracts from Potato Creek-A (AVM-negative), Woodlake and Parksville-B (AVM-positive) at 12.5 and 50 mg/ml, as well as a vehicle control (methanol), harvested at 4, 8, and 24h exposure, and washed once in cold phosphate buffered saline (PBS). Ten percent of the cell suspension was used for the Annexin V assay and the remaining 90% for the caspase assay. For the Annexin V assay, cells were then stained with fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide (PI) using the Vybrant Apoptosis Assay Kit #3 (Invitrogen, Carslbad, California, USA). Briefly, cells were washed once in cold PBS, re-suspended in annexin-binding buffer (10 mM HEPES, 140 mM $NaCl_2$, 2.5 mM $CaCl_2$, pH 7.4) to ~1 x 10^6 cells/ml, and stained with 5µl of FITC annexin V and 1 µg/ml of PI at room temperature for 15 minutes. Cells were analyzed on a Coulter EPICS XL flow cytometer immediately following incubation with excitation at 488 nm and emission wavelengths of 525 nm and 635 nm for FITC and PI, respectively. Cells showing only background fluorescence were determined to be live cells, FITC positive and PI negative cells were early apoptotic, and cells positive for both FITC and PI were late apoptotic or dead. Three independent experiments were conducted and the percentages of dead (PI^+) and apoptotic ($FITC^+/PI^-$) cells were compared by a repeated-measures one-way ANOVA followed by Tukey's multiple comparison test as performed by GraphPad Prism software. Due to

insufficient extract quantity only two replicates could be produced with the Parksville-A 50 mg/ml dose and it was not included in the statistical analysis.

To determine caspase activity, the cells were diluted in PBS to equivalent cell densities for all samples, centrifuged, and pellets stored at -80°C until analysis. Caspase activity was analyzed using the EnzChek Caspase-3 Assay Kit #1 (Invitrogen). The principle of this kit is the caspase activated cleavage of the 7-amino-4-methylcoumain-derived substrate Z-DEVD-AMC. Upon cleavage, this substrate yields a blue fluorescent product that can be measured as an indicator of caspase activity in the sample. Briefly, each pellet was re-suspended in cell lysis buffer and subjected to one freeze-thaw cycle to lyse cells. Following centrifugation, 50 µl of supernatant was transferred to individual wells of a 96-well plate. 50 µl of lysis buffer was used as a background control. 50 µl of substrate solution (0.2 mM Z-DEVD-AMC substrate; 10 mM dithiothreitol; 20 mM PIPES; 4 mM EDTA; 0.2% CHAPS) was added to each sample and control well and incubated 30 min at room temperature in the dark. Fluorescence was measured using a NOVOStar plate reader with excitation at 360 ±10 and emission detection at 450±10 nm. Background fluorescence (lysis buffer control wells) was subtracted from each experimental value. Three independent experiments were conducted and average fluorescence was compared among treatments by a repeated-measures one-way ANOVA followed by Tukey's multiple comparison test as performed by GraphPad Prism software.

Cytoskeletal Staining and Morphological Observations

F-actin. C6 cells were plated on poly-l-lysine coated coverslips at 1 x 10^5 cells/ml and allowed to attach overnight. Cells were dosed with Potato Creek-A (AVM-negative) and Woodlake (AVM-positive) crude methanol extracts at 12.5 mg/ml, as well as vehicle control (methanol only). Following exposure at 0, 1, 2, 4, 8, and 24 h, coverslips were washed twice in PBS, fixed in 3.7% formaldehyde for 10 min at room temperature, and permeabilized with 0.1% Triton X-100 for 3 min at room temperature. After two PBS washes, cells were stained with phalloidin (Oregon Green® 488 phalloidin, Invitrogen) plus 1% bovine serum albumin for 20 min. DAPI (4',6-diamidino-2-phenylindole) (0.25µg/ml) was added and incubated 5 min. Slides were washed twice in PBS and mounted on glass slides with Fluoromount G. Fluorescence, phase contrast, and differential interference contrast images were taken on an Olympus BX51 microscope with an Olympus DP70 digital camera (Olympus America, Inc., Central Valley, Pennsylvania, USA).

Tubulin. C6 cells were plated onto poly-l-lysine coated coverslips at 1 x 10^5 cells/ml and allowed to attach overnight. Cells were dosed with AVM-negative (Potato Creek-A, Potato Creek-B) and AVM-positive extracts (Woodlake and Parksville-A) at 12.5 mg/ml, as well as vehicle control (methanol). Following exposure for 0, 1, 2, 4, 8, and 24 h coverslips were washed twice in PBS, fixed in 4% paraformaldehyde 5 min at room temperature (RT), washed twice in PBS, and permeabilized with 100% methanol (-20°C) for 5 min. Coverslips were then

washed twice in PBS and incubated with 5% normal goat serum (Vector Laboratories, Inc., Burlingame, California, USA) in PBS for 45 min at 4°C, then incubated overnight at 4°C with primary antibody (monoclonal mouse anti-α-tubulin, Sigma-Aldrich, St. Louis, Missouri, USA) at 1:400 in PBS. The following day coverslips were washed twice in PBS and incubated 1h at RT with secondary antibody (anti-mouse IgG-FITC produced in goat, Sigma-Aldrich), washed twice in PBS, and stained with DAPI at 0.25 μg/ml for 5 min at RT in the dark. After a final PBS wash coverslips were mounted onto glass slides with Fluoromount G. Fluorescence, phase contrast, and differential interference contrast images were taken on an Olympus BX51 microscope on an Olympus BX51 microscope with an Olympus DP70 digital camera.

Cell Cycle Analysis by Flow Cytometry

Cell cycle analysis was conducted by measuring the DNA content of cells stained with propidium iodide. C6 cells were plated at ~5 x 10^5/ml in 6-well plates at 2 ml per well and allowed to attach overnight. Cells were exposed to vehicle control (methanol) and all AVM-negative and AVM-positive extracts (Table 1). In addition, cells were exposed to triethyltin bromide (97%, Acros Organics, New Jersey, USA) at 1.0, 5.0, 10.0, 20.0 and 50.0 μM, dissolved in methanol. Following exposure at 4, 8, and/or 24 h, cells were harvested with trypsin-EDTA (supernatant was also collected to include any detached cells), pelleted by centrifugation at 3500 rpm for 5 min, and fixed in ice-cold 70% ethanol overnight at -20°C. Cells were then pelleted (3500 rpm, 5 min, room

temperature) and stained with 10 µg/ml propidium iodide in PBS containing 10 µg/ml RNase for 1 h at room temperature in the dark. Flow cytometry analysis for cell cycle distribution was conducted on a Coulter EPICS XL with excitation at 488 nm and an emission wavelength of 635 nm. A minimum of 10,000 cells were counted. The percentage of cells in G_1, S, and G_2/M phases were estimated using Multicycle software (Phoenix Flow Systems, San Diego, California, USA). Three independent experiments were conducted and the percentages of cells in each phase were compared by a repeated-measures one-way ANOVA followed by Tukey's multiple comparison test as performed by GraphPad Prism software.

Results

Cytotoxicity Assays

Both Potato Creek-B and Parksville-A extracts reduced MTT conversion in a concentration-dependent manner for all cell lines (Figure 3), with complete cell death occurring at the highest concentration (100 mg/ml). Direct comparison of response to Potato Creek-B and Parksville-A extracts at each concentration revealed few significant differences (Figure 3). Calculated EC_{50} values (Table 6) did not differ significantly between the AVM-positive and AVM-negative extracts for C6 and PC-12 cells, but EC_{50}'s for Parksville-A were significantly lower than Potato Creek-B for N2a and QNR/D cells. N2a cells were the most sensitive cell line tested, with the lowest EC_{50} values for both extracts. LDH release in C6 cells was also induced in a concentration-dependent manner, with cytotoxicity reaching a plateau at approximately 90% at 25 mg/ml (Figure 4).

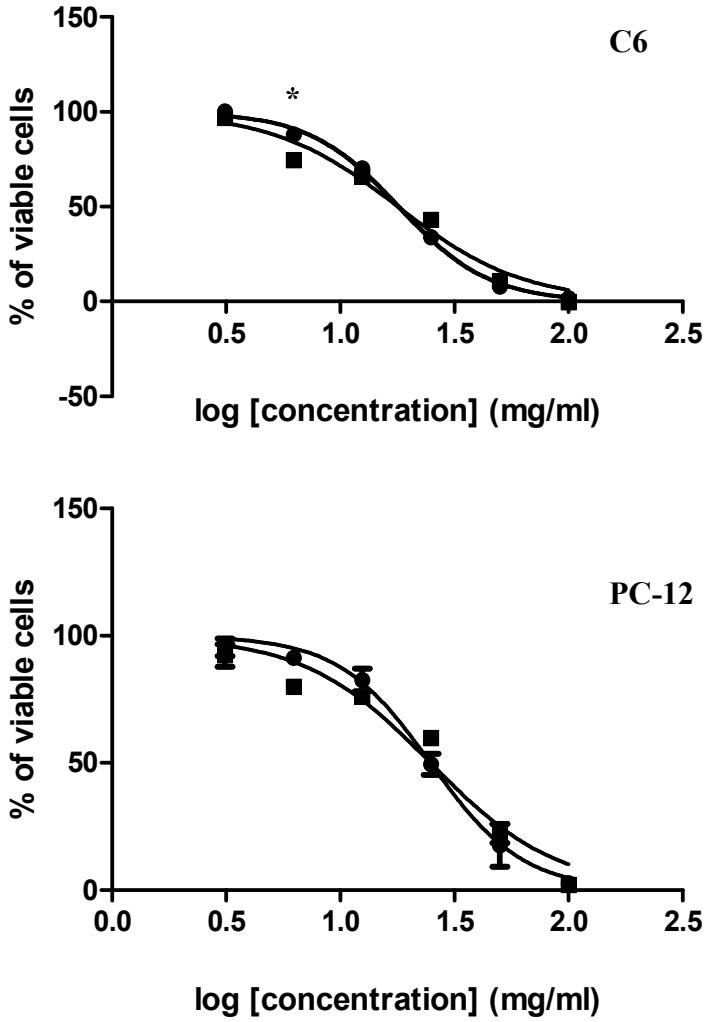

Figure 3. Effect of Potato Creek-B (■) and Parksville-A (●) crude methanol extracts on cytotoxicity of C6, PC-12, N2a, and QNR/D cell lines as determined by the MTT assay. Data are expressed as a percentage of viable cells relative to vehicle control (methanol only), with each point representing mean ± SEM of three or four independent experiments. Asterisks (*) signify a significant difference ($p < 0.05$) between the Potato Creek and Parksville extract for that concentration.

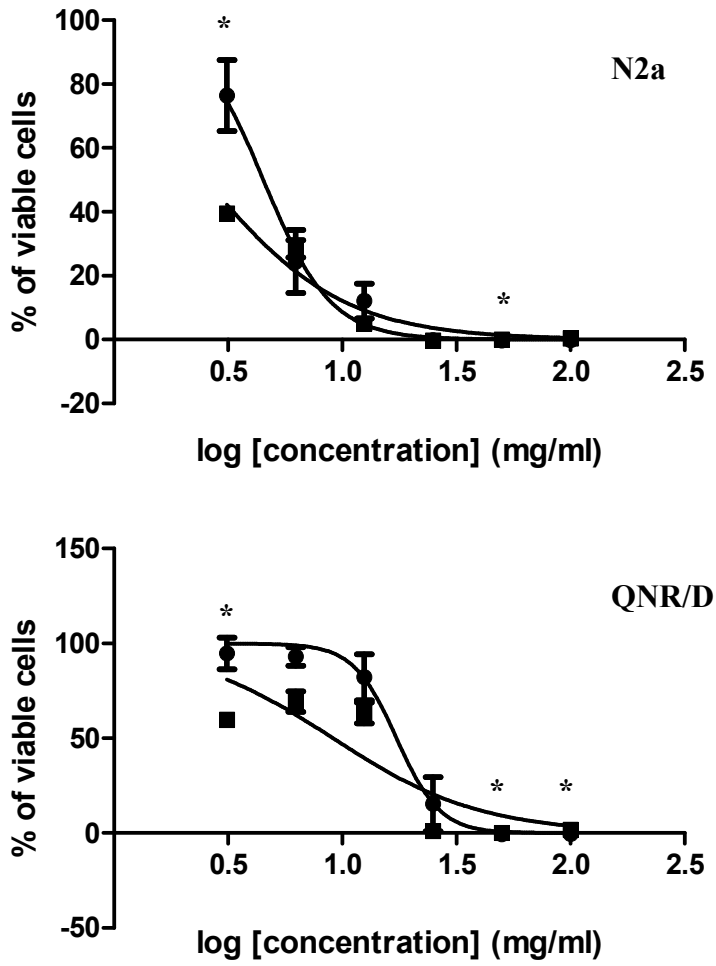

Figure 3. Effect of Potato Creek-B (■) and Parksville-A (●) crude methanol extracts on cytotoxicity of C6, PC-12, N2a, and QNR/D cell lines as determined by the MTT assay. Data are expressed as a percentage of viable cells relative to vehicle control (methanol only), with each point representing mean ± SEM of three or four independent experiments. Asterisks (*) signify a significant difference ($p < 0.05$) between the Potato Creek and Parksville extract for that concentration (Continued).

Table 6. Effective concentrations of crude extract that elicit half-maximal viability (EC_{50}) for each cell line following exposure for 24 h, as measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reduction assay or the lactate dehydrogenase (LDH) assay.

Cell line	Crude Extract EC_{50} (mg/ml) (mean ± SE)	
	Potato Creek-B (AVM-negative)	Parksville-A (AVM-positive)
MTT		
C6	18.15 ± 1.03	17.59 ± 1.13
PC-12	24.55 ± 1.06	25.32 ± 1.08
N2a	4.52 ± 1.08	2.51 ± 1.09*
QNR/D	17.30 ± 1.09	9.13 ± 1.17*
LDH		
C6	11.92 ± 1.12	14.50 ± 1.11

* Significantly different from corresponding Potato Creek-B value, $p < 0.05$.

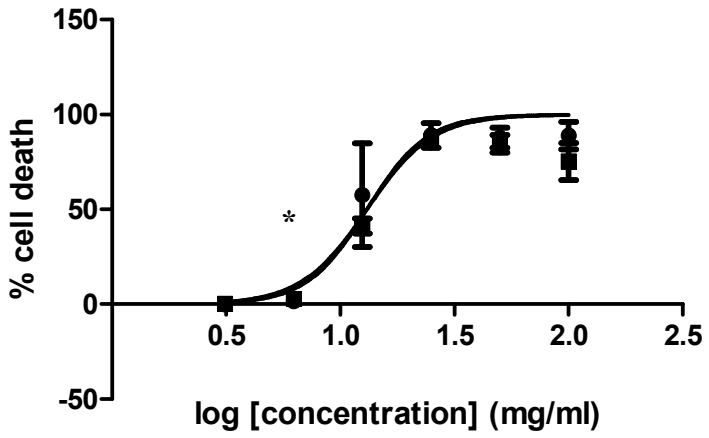

Figure 4. Effect of Potato Creek-B (■) and Parksville-A (●) crude methanol extracts on cytotoxicity of C6 cells as determined by the LDH assay. Data are represented as percent cell death, with each point representing mean ± SEM of three independent experiments. Asterisks (*) signify a significant difference ($p < 0.05$) between the Potato Creek and Parksville extract for that concentration.

The calculated LDH EC_{50} values (Table 6) for Parksville-A and Potato Creek-B were not significantly different.

Cell morphology was also noted at 4 and 24 h after exposure to the extracts in these experiments. In all cells lines the two high doses of extract contained large amounts of debris which obscured the cells and prevented any reliable observations. At the lower doses, a slight loss of cellular processes and increased rounding was noted in varying degrees with PC-12, N2a, and QNR/D cells in a concentration-dependent manner. This effect was observed in cells

exposed to both extracts, with the Parksville-A extract causing slightly greater changes in morphology but not being markedly different from Potato Creek-B. A more pronounced rounding effect was seen in C6 cells and was consistently observed at all concentrations of the Parksville-A extract but not in the Potato Creek-B extract. Small blebs could be seen on the cell surface of these rounded cells and it was hypothesized that these cells may be undergoing apoptosis. To test this hypothesis C6 cells were examined for markers of apoptosis and changes in cell morphology were also further explored by cytoskeletal staining, as described below.

Apoptosis Assays

To determine if the cell death observed in the cytotoxicity assays could be attributed to apoptotic mechanisms, C6 cells were exposed to AVM-positive (Parksville-A, Woodlake) and AVM-negative (Potato Creek-A) extracts and examined for markers of apoptosis. Cell surface expression of phosphatidylserine was measured using FITC-Annexin V, with propidium iodide used to distinguish cells in late apoptosis or necrosis. The percentage of apoptotic cells ($FITC^+/PI^-$) in populations exposed to AVM-negative and AVM-positive extracts did not differ significantly from control populations at any time point and consisted of a very small fraction of the total cell population (<2%). The percentage of dead/late apoptotic cells (PI^+) did not differ from control populations at the low dose of any extract, nor did it change over time. However, the high dose of Potato Creek-A and Woodlake extracts induced a significant rise in PI^+ cells at 24 h (Figure 5).

The Annexin V assay is unable to differentiate cells present in late apoptosis or necrosis, since both populations will stain with PI and FITC. Therefore, while there is an increase in the number of dead cells in the population with the high dose of extract, it is not clear by which mechanism these cells died. Since the percentage of $FITC^+/PI^-$ cells remained low throughout the experiment and did not differ between samples it is reasonable to assume that the majority of the dead cells did not undergo apoptosis.

As a second measure of apoptosis, caspase-3 activity was monitored in C6 cells at 4, 8, and 24 h after exposure to these extracts. Potato Creek-A and Woodlake extracts both resulted in decreased caspase activity compared to control populations at 8 and 24 h with the high dose (50 mg/ml) (Figure 6). No other significant differences were observed. The decrease in caspase activity seen at the high doses of extracts may be due to increased cell death in these populations, as was observed in the Annexin assay. Cell death was not preceded by an increase in caspase activity. Taken together, these data indicate that neither the AVM-positive nor AVM-negative extracts significantly induce apoptosis in C6 cells at the concentrations and time points examined.

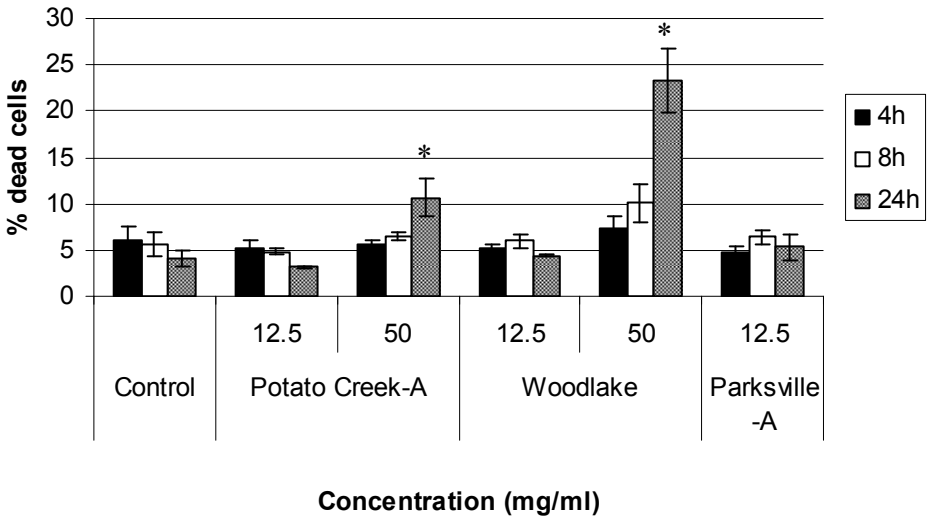

Figure 5. Dead/late apoptotic C6 cells as indicated by staining with propidium iodide following exposure to crude methanol extracts at 12.5 and 50 mg/ml for 4, 8, or 24 h. Data represent means ± SEM for 3 independent experiments. Asterisk (*) indicates significant difference ($p < 0.05$) from control.

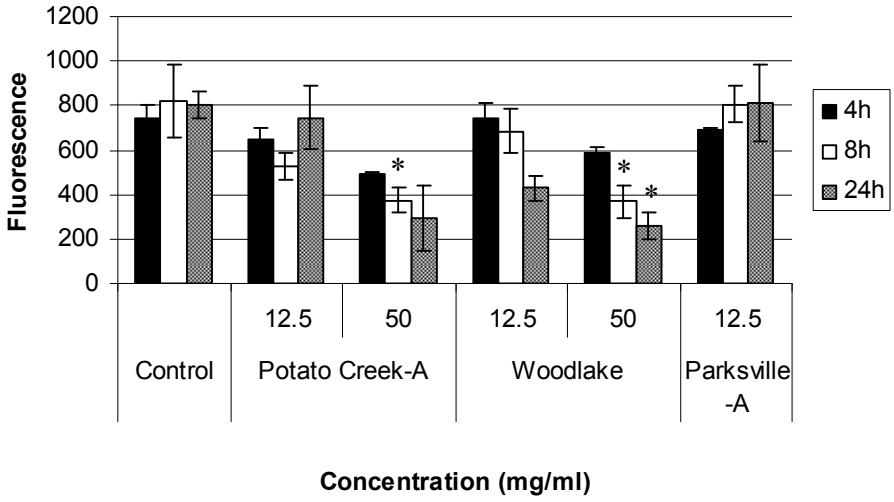

Figure 6. Caspase-3 activity in C6 cells following exposure to crude methanol extracts at 12.5 and 50 mg/ml for 4, 8, or 24 h. Data represent means ± SEM for 3 independent experiments. Asterisk (*) indicates significant difference ($p < 0.05$) from control.

Cytoskeletal Staining and Morphological Observations

To further investigate the morphological changes noted in the cytotoxicity assays, C6 cells were exposed to Potato Creek-A and Woodlake extracts as well as vehicle control for 0, 1, 2, 4, 8, and 24 h, stained for F-actin and α-tubulin and viewed under fluorescent and light microscopy. Control cells varied in morphology from flat, polygonal cells with no or varying numbers of processes to elongated bipolar cells. Phalloidin staining revealed organized microfilament networks in the majority of cells, consisting of parallel bundles of filaments (Figure 7). Spiky filopodia were seen protruding from the cell surface of many

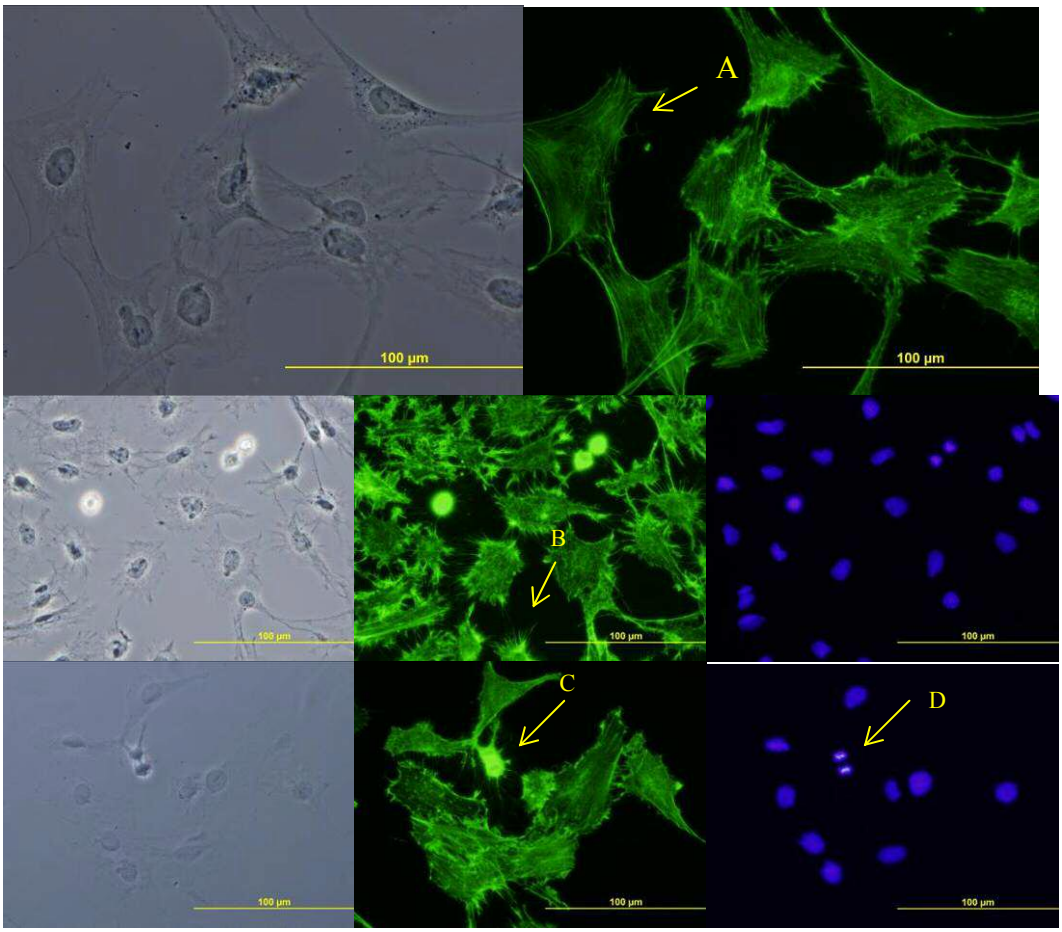

Figure 7. Phase contrast and fluorescent images of C6 cells exposed to vehicle control (methanol) for 4 h and stained to visualize F-actin (green) and DNA (blue). Organized parallel bundles (A) and spiky filopodia (B) were present. Rounded, mitotic cells with intense actin staining (C) and condensed chromosomes (D) were also observed.

cells (Figure 7). A small number of rounded cells were also present with intense actin staining, especially at the periphery (Figure 7). Parallel DAPI staining revealed condensed chromosomes in the nuclei of these rounded cells, indicating these cells were undergoing mitosis (Figure 7). Control cell morphology did not change during the 0-8 h time points, but the percentage of elongated, bipolar cells was greatly increased at 24 h and the cell density was also increased (Figure 8). Tubulin staining revealed normal, organized microtubule networks in interphase cells and normal spindle apparatus in mitotic cells (Figure 9).

Cells exposed to Potato Creek-A extract did not appear to differ from control cells in morphology, microfilament or microtubule organization, or in the percentage of cells undergoing cell division (Figures 8, 10, 11). However, cells treated with Woodlake extract experienced an increase in the proportion of rounded cells beginning as early as 1 h after exposure and continuing through 24 h (Figure 8). DAPI staining revealed condensed chromosomes in the nuclei of these cells, but unlike control and Potato Creek-B extract treated cells the chromosomes were consistently observed only in the beginning stages of mitosis and did not appear to move past prophase (Figures 12, 13). F-actin organization in interphase cells appeared to be normal (Figure 12), with the exception of the 24 h time point in which few cells remained in the normal morphology and those that had not yet rounded had less organized parallel bundles. In addition, fewer processes were seen in cells at the latter time points and there was an increase of the amorphous actin concentrations at the cell periphery (Figure 12), which may represent processes retracting as cells began to round. Tubulin staining revealed

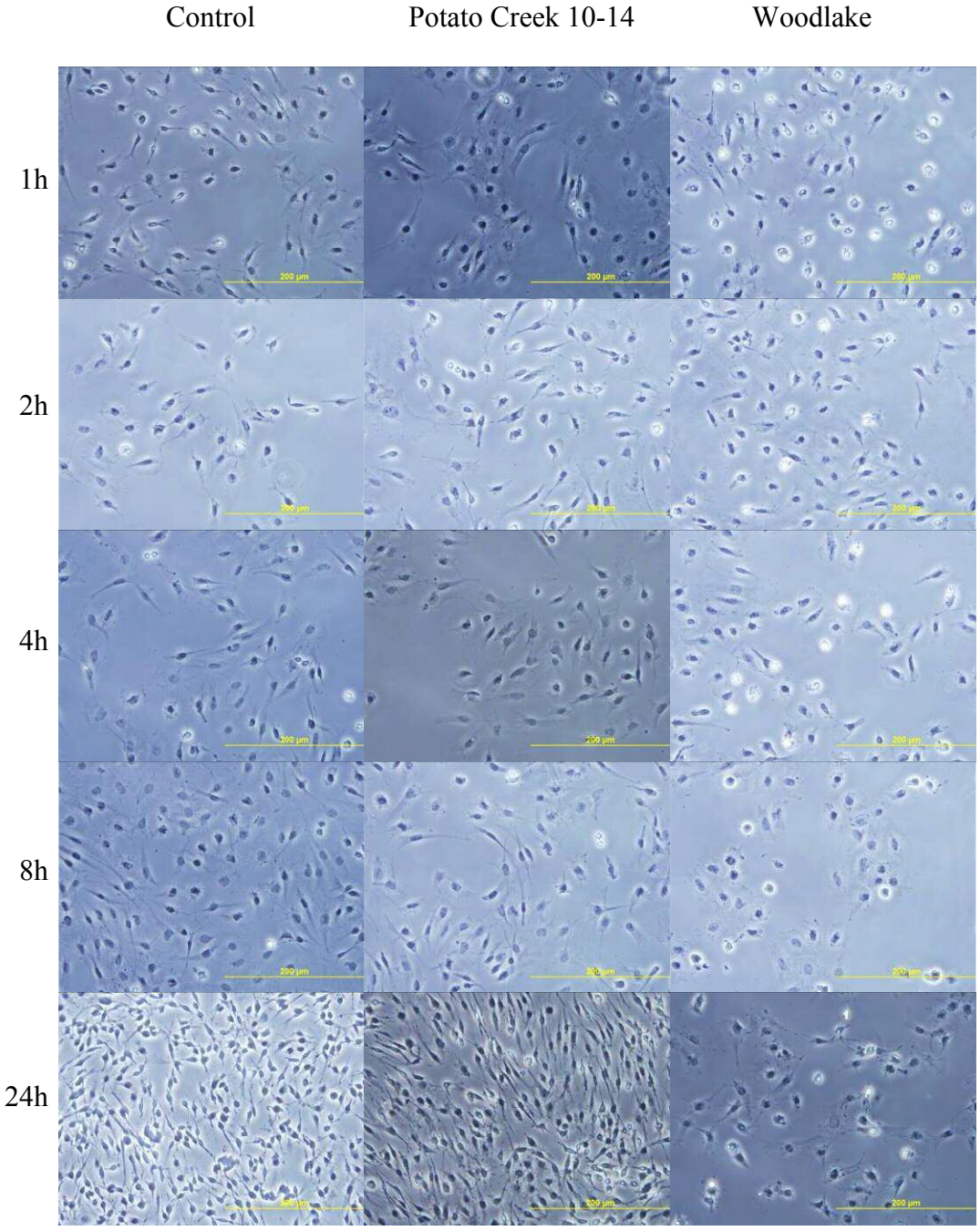

Figure 8. Phase contrast images of C6 cells exposed to vehicle control (methanol) and crude methanol extracts from an AVM-negative (Potato Creek-A) and AVM-positive (Woodlake) site.

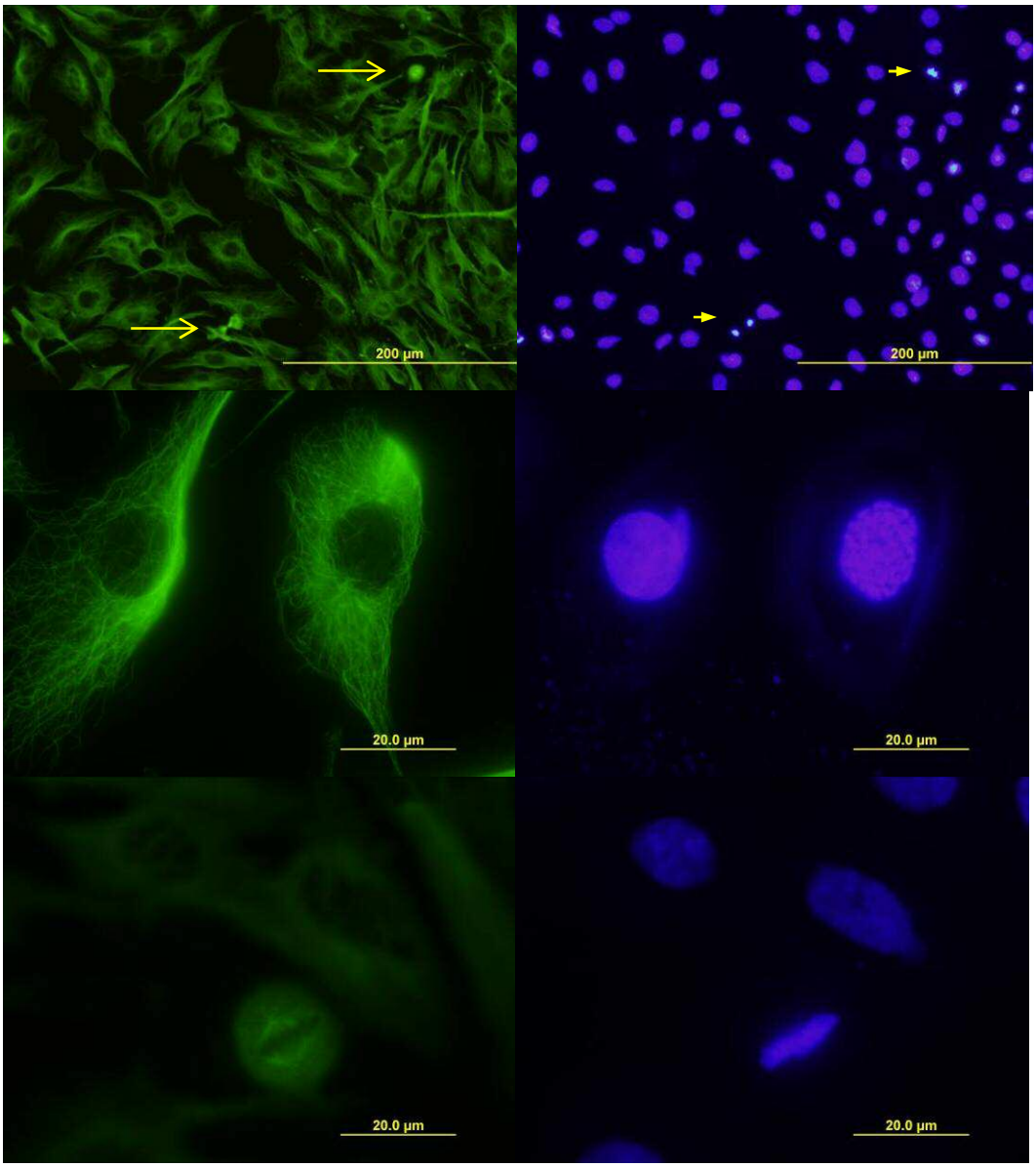

Figure 9. Fluorescent images of C6 cells exposed to vehicle control (methanol) for 4 h and stained to visualize microtubules (green) and DNA (blue). Normal mitotic spindles (arrows) and chromosomes (arrowheads) can be seen in various stages of mitosis.

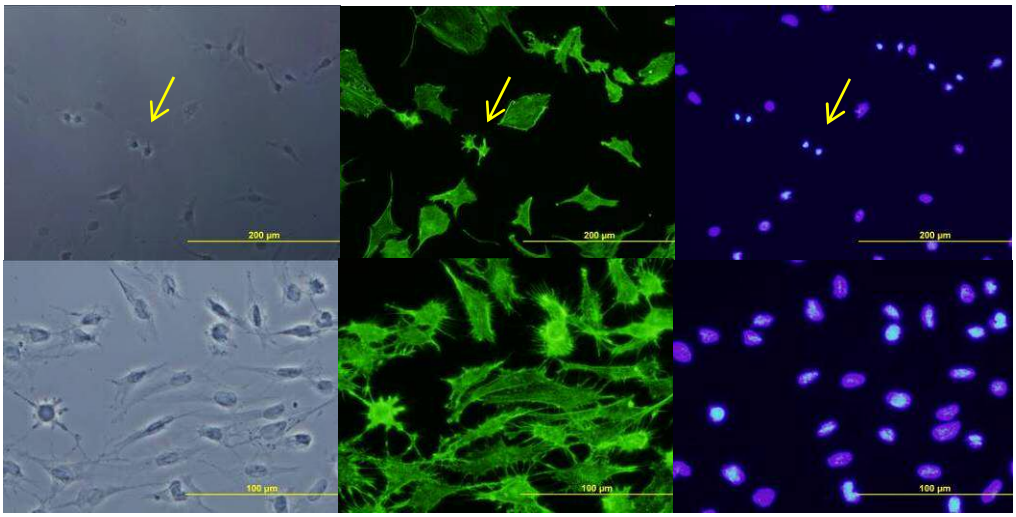

Figure 10. Phase contrast and fluorescent images of C6 cells exposed to crude methanol extract from an AVM-negative site (Potato Creek-A) for 4 h. Cells were stained to visualize F-actin (green) and DNA (blue). Mitotic cells can be seen dividing (arrows).

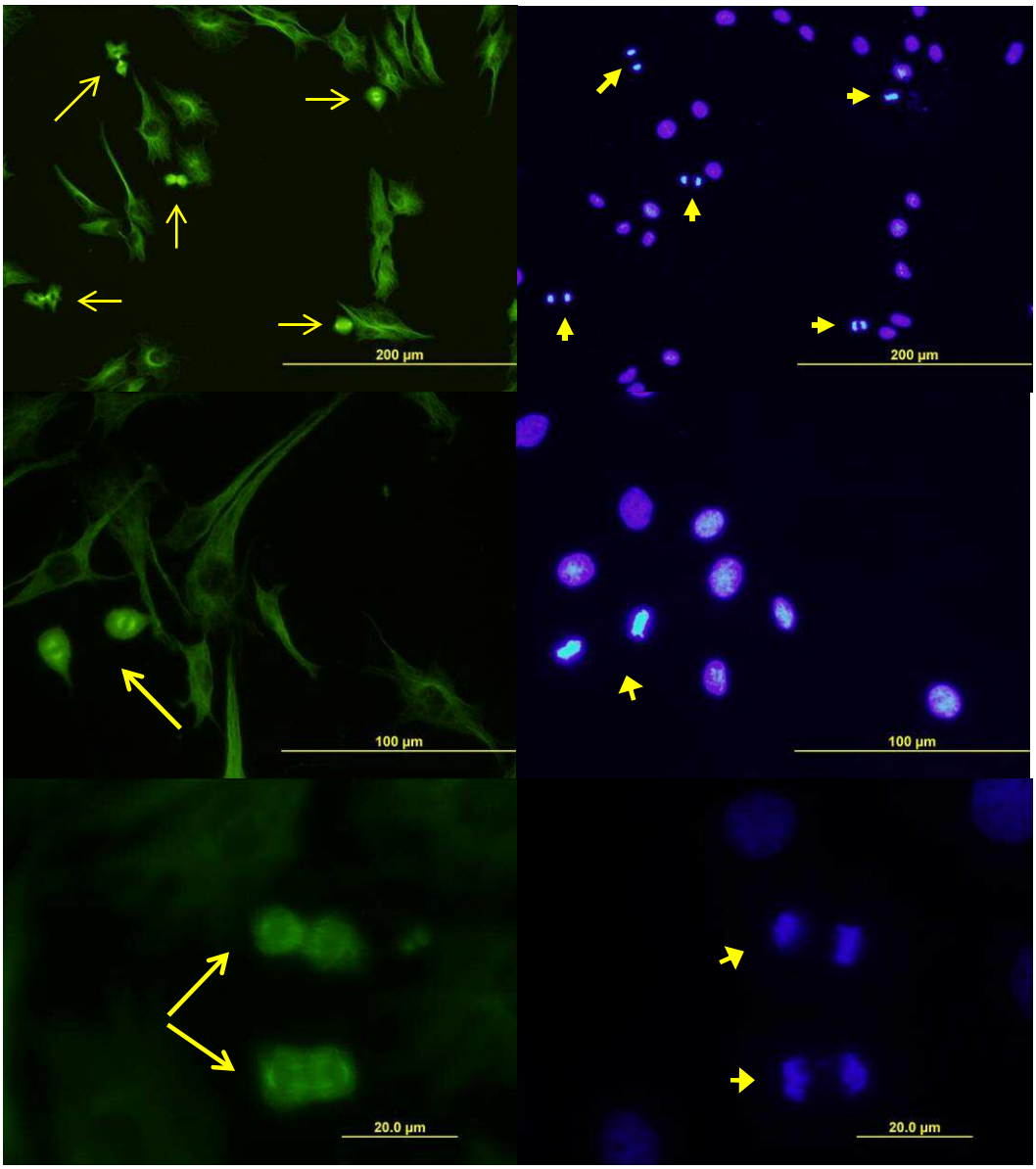

Figure 11. Fluorescent images of C6 cells exposed to crude methanol extract from an AVM-negative site (Potato Creek-A) for 4 h and stained to visualize microtubules (green) and DNA (blue). Normal mitotic spindles (arrows) and chromosomes (arrowheads) can be seen in various stages of mitosis.

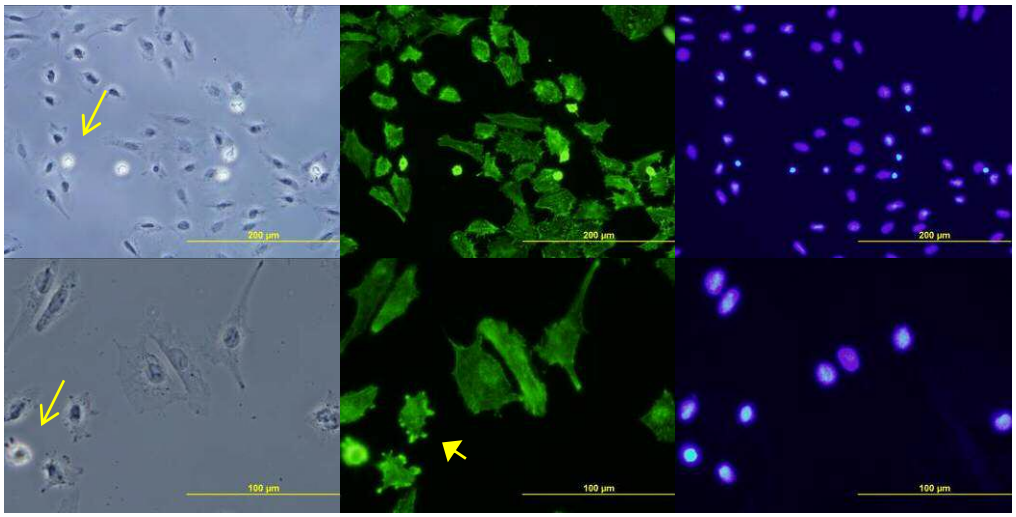

Figure 12. Phase contrast and fluorescent images of C6 cells exposed to crude methanol extract from an AVM-positive site (Woodlake) for 4 h. Cells were stained to visualize F-actin (green) and DNA (blue). Rounded cells (arrows) have condensed chromosomes and are thought to be present in early mitosis. Amorphous accumulations of actin (arrowhead) at the cell periphery may represent processes retracting as cells round.

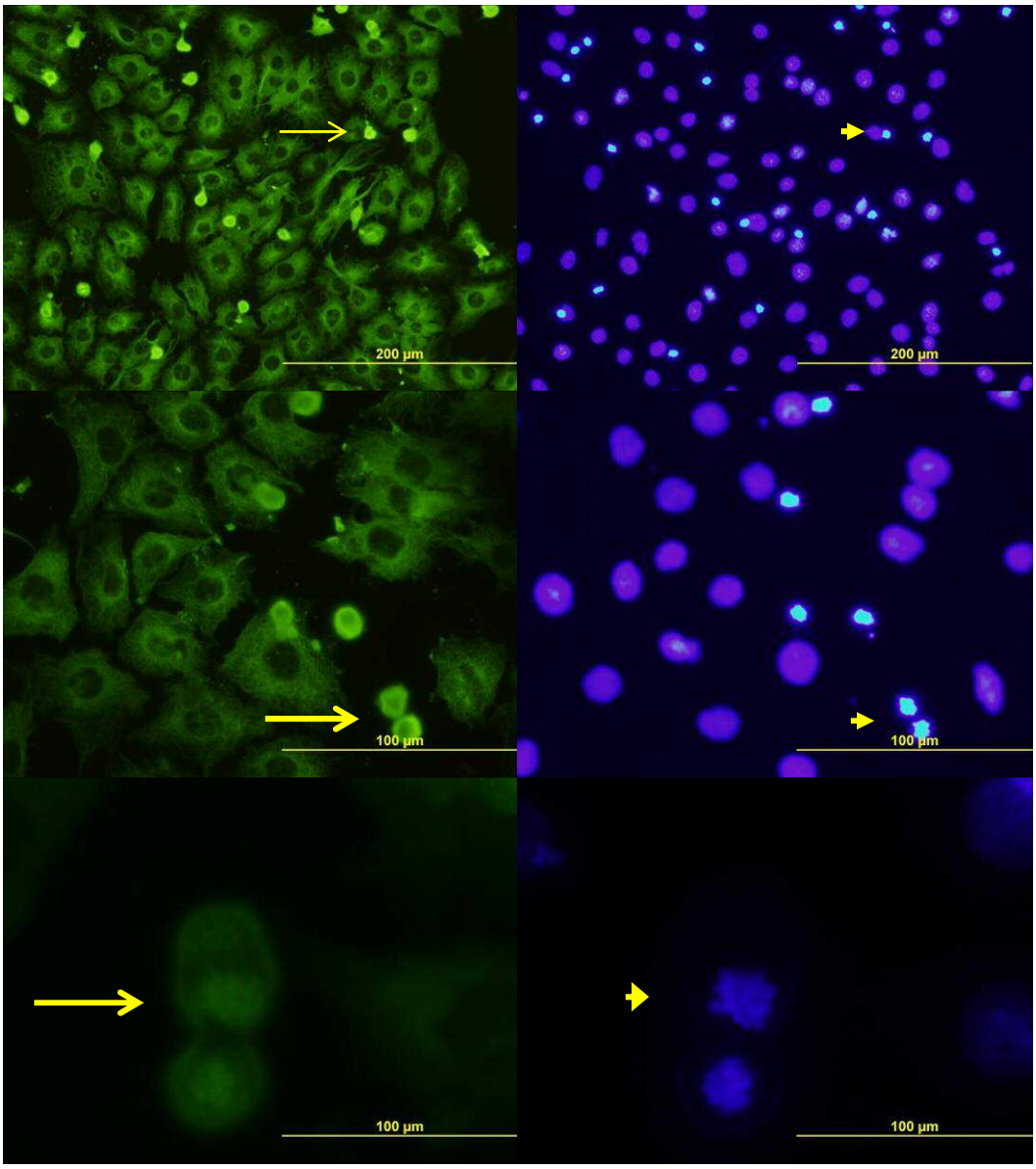

Figure 13. Fluorescent images of C6 cells exposed to crude methanol extract from an AVM-positive site (Woodlake) for 4 h and stained to visualize microtubules (green) and DNA (blue). Cells are uniformly present in prophase or prometaphase as condensed chromosomes can be seen (arrowheads), but there is no corresponding mitotic spindle formation (arrows).

normal microtubule networks in interphase cells but the normal spindle apparatus was absent or poorly defined in the majority of mitotic cells (Figure 13). Normal spindle asters could be seen in some cells at the earlier time points (1 and 2 h post exposure), but at 4, 8, and 24h the majority of mitotic cells contained no visible spindle apparatus and no clear organization of microtubules (Figure 13). Taken together, the cytoskeletal stains suggest the Woodlake extract slows or blocks progression from prophase to metaphase. An additional AVM-positive (Parksville-A) and AVM-negative (Potato Creek-B) extract were subsequently tested on C6 cells and stained for tubulin at 4, 8, and 24 h. Cells exposed to Potato Creek-B did not differ from control cells. Parksville-A induced a similar effect to Woodlake, with an increase in rounded, mitotic cells and an absence of spindle microtubules.

Cell Cycle Analysis

To further investigate the potential mitotic block revealed with the cytoskeletal staining, a cell cycle analysis was conducted on several extracts from both AVM-positive and AVM-negative sites. C6 cells were exposed to the extracts at 12.5 and 50 mg/ml for 4, 8, and 24 h and the percentage of cells in G_1, S, and G_2/M was determined by flow cytometry. Percentages of cells in each phase are displayed in Table 7 (AVM-negative) and Table 8 (AVM-positive) and representative histograms are shown in Figures 14 and 15. AVM-positive extracts consistently produced an increase in the percentage of cells in S-phase or G_2/M phase compared to control populations, and a corresponding decrease of cells in

G_1. The high dose (50 mg/ml) generally resulted in an increase in S-phase cells, while the low dose (12.5 mg/ml) produced an increase of cells in G_2/M. This increase could be seen as early as 4 h, and with the high dose did not change at the 8 or 24 h time points. At the low dose, depending on the extract, the percentage of cells in G_2/M that were initially increased at 4 h either continued to increase at 8 and 24 h (DeGray, Parksville-A) or decreased and returned to near control levels by 24 h (Woodlake, Parksville-B). The presence of a broad sub-G_1 peak, most likely representing cellular fragments and debris in the high doses of extract, indicates an increase in cell death at these concentrations.

Cells exposed to extracts from AVM-negative sites at the low dose were not significantly different than control cell populations at any time point. The high dose of Potato Creek-A and Potato Creek-B produced a slight but not significant increase in the proportion of S-phase and G_2/M cells at all time points. The high dose of SCWMA extract cause a significant increase in the proportion of cells in G_2/M phase at 4 and 8 h, but populations returned to near control levels by 24 h.

Because the cell cycle analysis revealed differences in the extracts of the AVM-negative and AVM-positive sites tested, additional extracts from one AVM-positive site and two AVM-negative sites were prepared and analyzed by this assay to determine if the results would remain consistent. For these analyses cells were exposed to the extracts for four hours only, since this time point was adequate in detecting the cell cycle disruption in the initial tests and the effect was often lost at the later time points. Results are shown in Table 9 and Figure 16. The AVM-positive extract (Petersburg) induced a significant increase of G_2/M

Table 7. Cell cycle distribution of C6 cells exposed to AVM-negative extracts from Potato Creek Embayment, South Carolina and the Santee Cooper Wildlife Management Area (SCWMA), South Carolina and vehicle control (methanol) for 4, 8, or 24 h.

	4 h			8 h			24 h		
	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
Control	47.30 ± 4.88	31.97 ± 3.10	20.73 ± 1.77	59.33 ± 2.87	28.97 ± 3.09	11.70 ± 0.96	75.23 ± 2.76	18.10 ± 1.25	6.67 ± 1.56
Potato Creek A									
12.5 mg/ml	46.73 ± 5.40	32.27 ± 3.32	21.03 ± 2.27	58.73 ± 2.63	29.30 ± 4.06	12.00 ± 1.60	69.83 ± 4.09	21.17 ± 2.04	9.03 ± 2.07
50.0 mg/ml	42.10 ± 7.16	36.17 ± 6.13	21.73 ± 1.58	53.37 ± 4.81	26.27 ± 2.81	20.37 ± 2.34	64.43 ± 4.17	22.83 ± 6.61	12.73 ± 2.70
Potato Creek B									
12.5 mg/ml	50.83 ± 8.29	30.87 ± 5.55	18.30 ± 2.99	57.80 ± 2.43	29.70 ± 3.75	12.47 ± 1.71	67.70 ± 6.33	21.37 ± 2.65	10.90 ± 3.75
50.0 mg/ml	43.67 ± 5.97	34.33 ± 5.06	22.00 ± 0.96	54.60 ± 4.37	25.90 ± 2.00	19.50 ± 3.02	63.27 ± 6.89	22.77 ± 2.88	13.97 ± 4.02
SCWMA									
12.5 mg/ml	47.40 ± 5.28	32.47 ± 3.52	20.13 ± 2.03	62.07 ± 2.23	27.27 ± 3.29	10.70 ± 1.47	75.23 ± 1.74	17.97 ± 0.62	6.80 ± 1.12
50.0 mg/ml	31.70 ± 5.01*	34.10 ± 6.24	34.17 ± 2.29*	36.10 ± 8.30*	28.77 ± 4.34	35.13 ± 6.91*	67.30 ± 2.07	13.80 ± 1.80	18.90 ± 2.69

* Significantly different from control value, $p < 0.05$.

Table 8. Cell cycle distribution of C6 cells exposed to AVM-positive extracts from Parksville Cove (J. Strom Thurmond Lake), South Carolina; Woodlake, North Carolina; DeGray Lake, Arkansas; and vehicle control (methanol) for 4, 8, or 24 h.

	4h			8h			24h		
	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
Control	47.30 ± 4.88	31.97 ± 3.10	20.73 ± 1.77	59.33 ± 2.87	28.97 ± 3.09	11.70 ± 0.96	75.23 ± 2.76	18.10 ± 1.25	6.67 ± 1.56
Parksville-B									
12.5 mg/ml	33.07 ± 4.81*	31.47 ± 3.00	35.47 ± 1.94*	45.50 ± 3.94	29.87 ± 2.71	24.67 ± 2.74	64.00 ± 3.79	21.27 ± 2.02	14.73 ± 2.19
50.0 mg/ml	29.57 ± 2.98*	49.83 ± 5.23*	20.63 ± 3.27	28.67 ± 2.62*	50.13 ± 4.51	21.23 ± 2.28	27.57 ± 2.23*	48.17 ± 5.78*	24.27 ± 3.63*
Woodlake									
12.5 mg/ml	27.83 ± 4.67*	34.43 ± 3.16	37.73 ± 1.75*	38.00 ± 8.35*	34.47 ± 8.29	27.53 ± 4.53*	59.23 ± 2.34	20.77 ± 1.00	20.03 ± 1.56
50.0 mg/ml	26.90 ± 2.17*	49.43 ± 3.96*	23.67 ± 1.78	26.53 ± 1.83*	49.43 ± 5.48	24.10 ± 3.86	26.20 ± 0.92*	45.63 ± 5.15*	28.20 ± 4.28*
DeGray									
12.5 mg/ml	19.67 ± 1.43*	36.80 ± 3.78	43.57 ± 2.92*	10.20 ± 0.30*	29.87 ± 2.37	59.93 ± 2.02*	10.03 ± 0.33*	15.27 ± 1.16	74.73 ± 0.88*
50.0 mg/ml	25.27 ± 1.07*	42.07 ± 1.85*	32.67 ± 0.98*	15.97 ± 2.18*	48.67 ± 5.36	35.37 ± 4.62*	20.33 ± 2.95*	34.53 ± 8.63	45.13 ± 7.11*
Parksville-A									
12.5 mg/ml	20.00[a]	26.20	53.80	15.30	23.90	60.90	32.60	18.80	48.60

[a]Values represent only one replicate and are therefore not included in statistical analysis.

* Significantly different from control value, $p < 0.05$.

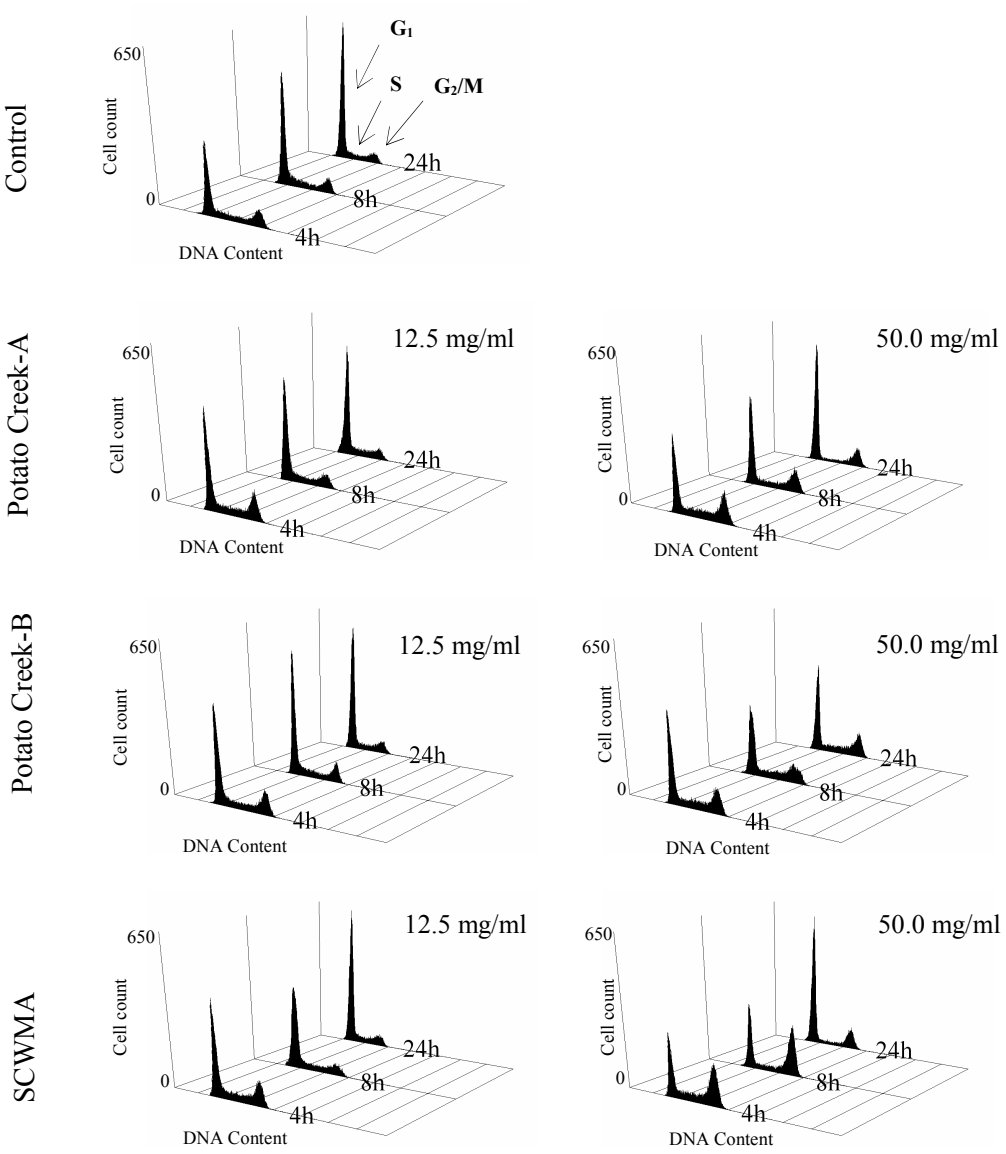

Figure 14. Representative histograms of cell cycle distribution in C6 cells treated with crude hydrilla extracts from AVM-negative sites and analyzed 4, 8 and 24 h after exposure. Total DNA content is measured as the intensity of propidium iodide fluorescence. The experiment was repeated three times with similar results.

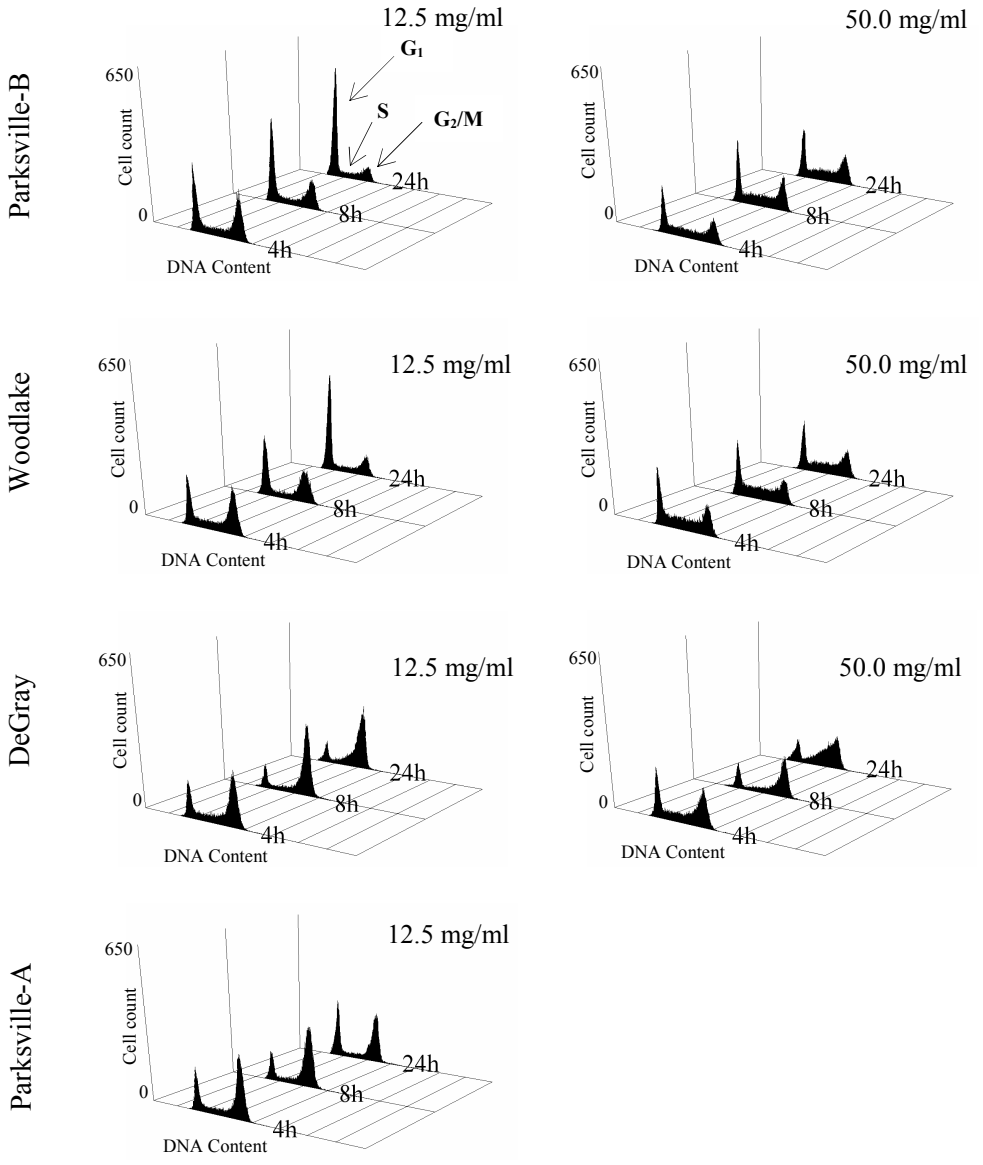

Figure 15. Representative histograms of cell cycle distribution in C6 cells treated with crude hydrilla extracts from AVM-positive sites and analyzed 4, 8 and 24 h after exposure. Total DNA content is measured as the intensity of propidium iodide fluorescence. The experiment was repeated three times with similar results.

Table 9. Cell cycle distribution of C6 cells exposed to AVM-positive (Petersburg Cove, South Carolina) and AVM-negative (Dens Swamp, South Carolina; Lake Istokpoga, Florida) extracts and a vehicle control (methanol) for 4h.

	G_1	S	G_2/M
Control	58.33 ± 2.97	30.77 ± 2.59	10.87 ± 1.22
Petersburg			
12.5 mg/ml	36.80 ± 0.10*	33.57 ± 2.84	29.63 ± 2.94*
50.0 mg/ml	54.00 ± 4.51	30.53 ± 0.84	15.47 ± 4.05
Dens Swamp			
12.5 mg/ml	60.87 ± 1.37	28.20 ± 1.11	10.93 ± 1.19
50.0 mg/ml	53.83 ± 1.02	31.23 ± 1.19	14.90 ± 1.42
Istokpoga			
12.5 mg/ml	59.53 ± 1.43	30.23 ± 2.28	10.23 ± 1.79
50.0 mg/ml	60.23 ± 0.79	28.57 ± 1.77	11.20 ± 1.83

*Significantly different from control value, $p < 0.05$.

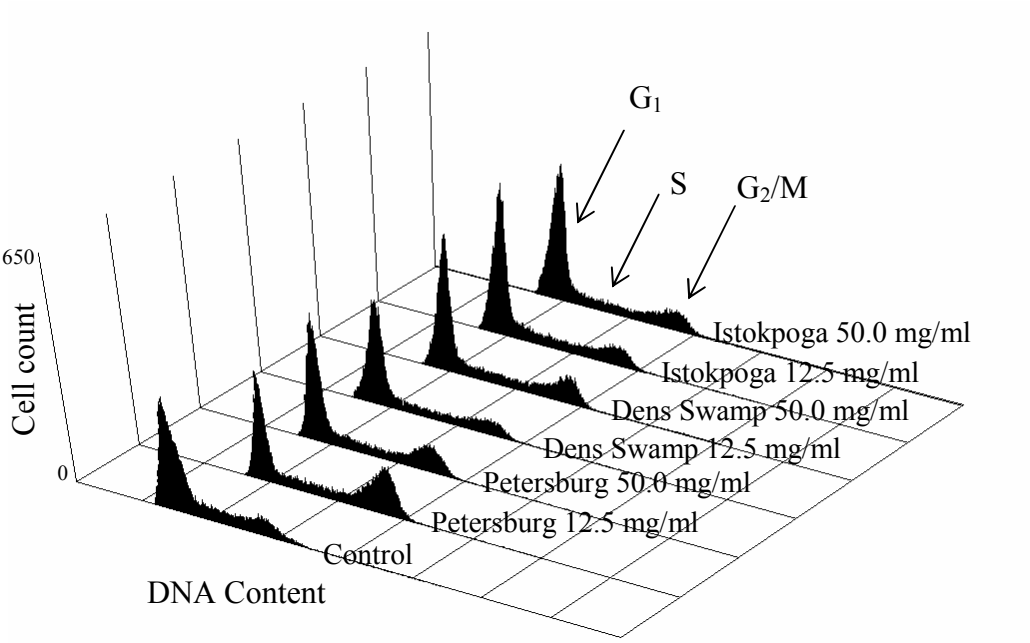

Figure 16. Representative histograms of cell cycle distribution in C6 cells treated with crude hydrilla extracts and analyzed 4 h after exposure. Total DNA content is measured as the intensity of propidium iodide fluorescence. The experiment was repeated three times with similar results.

phase cells at the low dose and did not differ from control populations at the high dose. A large sub-G_1 peak indicating cell death was also seen at the high dose. Neither AVM-negative extract induced changes in cell cycle distribution at the low dose, while one (Dens Swamp) induced a slight but not significant increase in G_2/M cells at the high dose.

In an attempt to determine if similar cell cycle disruption could be seen in a known compound causing similar effects to AVM *in vivo*, and to better understand the dose-response for the cell cycle results, cells were exposed to a dilution series of triethyltin (TET). Results are seen in Table 10 and Figure 17 and are similar to those observed with the crude extracts. At 1.0 μM, TET did not differ significantly from control values, although there was a slight increase in G_2/M at 4 h with one replicate. At 5.0 μM, TET caused a significant increase of cells in G_2/M that continued through 24 h. An increase in S-phase and G_2/M cells resulted from 10 μM TET and did not change notably over the time points, similar to that observed in the high dose of AVM-positive extracts. The highest concentrations of TET (20, 50 μM) caused a slight but not significant increase in S and G_2/M cells at 4 h, with no subsequent changes at the later time points, leading to significantly more cells in S-phase than that of controls by 24 h. The sub-G1 peaks indicating cell death also increased with the higher doses.

Table 10. Cell cycle distribution of C6 cells exposed to triethyltin for 4, 8, or 24 h.

	4h			8h			24h		
	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
Control	47.30 ± 4.88	31.97 ± 3.10	20.73 ± 1.77	59.33 ± 2.87	28.97 ± 3.09	11.70 ± 0.96	75.23 ± 2.76	18.10 ± 1.25	6.67 ± 1.56
1.0 µM	52.23 ± 3.23	28.40 ± 1.82	18.37 ± 3.43	68.37 ± 1.64	19.50 ± 0.58	12.10 ± 1.95	80.03 ± 2.30	14.77 ± 0.37	5.20 ± 1.93
5.0 µM	41.07 ± 5.69	35.93 ± 4.11	22.97 ± 3.79	33.93 ± 7.11*	29.37 ± 10.50	36.70 ± 6.31*	28.40 ± 4.92*	28.20 ± 3.43	43.37 ± 4.39*
10.0 µM	35.23 ± 6.69	41.53 ± 10.13	23.27 ± 6.12	32.30 ± 4.59*	39.30 ± 7.90	28.43 ± 4.69	29.07 ± 3.54*	40.27 ± 5.36*	30.63 ± 2.31*
20.0 µM	53.57 ± 2.29	35.83 ± 5.56	10.60 ± 3.41	55.87 ± 2.46	29.00 ± 3.76	18.47 ± 2.74	49.40 ± 1.67*	32.87 ± 2.03*	17.73 ± 3.43
50.0 µM	51.00 ± 2.02	34.60 ± 0.50	14.43 ± 2.10	49.70 ± 1.82	36.00 ± 0.50	14.37 ± 2.25	45.17 ± 2.02*	41.10 ± 0.92*	13.67 ± 1.48

* Significantly different from control value, $p < 0.05$.

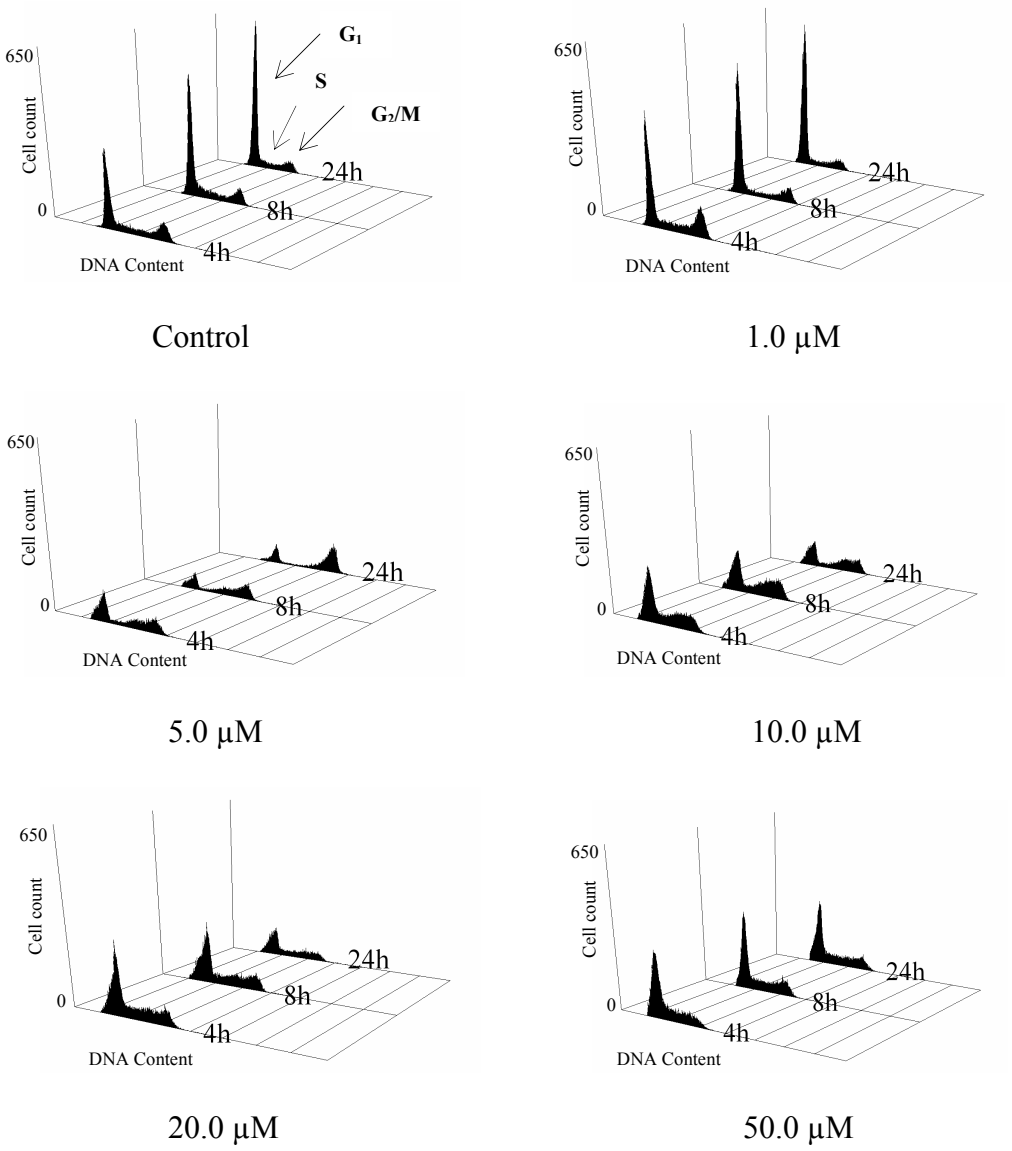

Figure 17. Representative histograms of cell cycle distribution in C6 cells treated triethyltin and analyzed 4, 8, and 24 h after exposure. Total DNA content is measured as the intensity of propidium iodide fluorescence. The experiment was repeated three times with similar results.

Discussion

AVM-positive extracts caused a significant disruption of the cell cycle in C6 cells. At lower concentrations, the extracts seem to slow progression through mitosis, causing an increase of cells in G_2/M at 4 h. This effect is then either alleviated after 24 h, presumably at lower concentrations, or cells continue to accumulate in G_2/M. Higher concentrations cause a buildup of cells is S-phase as well as G_2/M at 4 h, with no subsequent changes occurring through 24 h. The high concentrations of AVM-positive extracts were also associated with an increase in cell death. TET caused a similar cell cycle arrest. Lower, sub-lethal concentrations of both TET and AVM-positive extracts caused an increase in G_2/M phase cells while higher concentrations resulting in cell death induce more of an increase in S-phase cells. The highest concentrations do not appear to significantly affect the cell cycle, most likely because these concentrations result in acute cell death (20 and 50 μM TET, 50 mg/ml Petersburg extract). AVM-negative extracts also caused a slight increase in G_2/M cells, but were not nearly as efficient at inducing this effect as AVM-positive extracts, with only one extract (SCWMA) inducing a significant change in cell cycle distribution at only the high concentration.

The cell cycle assay appears to be a useful tool in identifying extracts from AVM-positive sites, although it is unknown whether or not the cell cycle arrest is caused by the etiologic agent of AVM. An increase in G_2/M cells was also noted in C6 cells exposed to AVM-negative extracts, although the effect was consistently lower than that produced with AVM-positive extracts and all

populations were recovered by 24 h. It is possible that some of the AVM-negative extracts tested could have contained the AVM agent in small amounts. For instance, the SCWMA extract, which induced a significant G_2/M arrest at 4 h, came from a site with no known history of AVM, but the vegetation had not been previously tested by *in vivo* bioassay to confirm that the AVM agent was not present. The two extracts from Potato Creek, which had been previously tested by mallard bioassay and did not induce AVM lesions, also induced a very slight increase in G_2/M cells. It could be that these samples did contain low levels of the causative agent and that the cell cycle analysis is a more sensitive measure than *in vivo* assays. The Stigonematalan cyanobacteria that is suspected to be the source of the AVM toxin was not present in the vegetation collected from AVM-negative sites, as confirmed by microscopic analysis and PCR (Williams, unpubl. data). However, despite the strong correlations between this cyanobacteria and AVM-positive reservoirs (Wilde et al., 2005) a direct causal relationship between this cyanobacterium and AVM has not yet been established and it is possible that this cyanobacterium is not involved. If the etiologic agent of AVM was not present in the vegetation collected from the AVM-negative sites then the observed cell cycle arrest could be due to another unrelated compound present in the crude extract. Another possibility is that the AVM toxin could be having an additive or synergistic effect with some other compound. Isolation of the AVM toxin from the crude extract will be necessary to further evaluate these results.

Cell cycle arrest has been noted in at least one compound known to produce intramyelinic edema –TET. Zucker et al. (1989) found that TET induced

a cell cycle block in G_2/M at or above 5 μM in murine erythroleukemic cells after 4 h of exposure. Similar results were found in this study, with TET inducing increases in both G_2/M and S-phase cells at 5 and 10 μM. In addition, the TET derivative triethyltin(IV)lupinylsulfide hydrochloride, which was synthesized and tested as a potential anticancer drug, induced a 42% increase in G_2/M cells at 24 h in HCT-8 human colon carcinoma cells (Barbieri et al., 2001). The mechanism behind these effects has not been elucidated.

The increase in S-phase cells at higher concentrations of extract appears to coincide with the increase in cytotoxicity. S-phase is the period of DNA synthesis and reports of S-phase arrest are less frequent than that of G_2/M or G_1 arrest in the literature on cell cycle disruption. S-phase arrest or delay has been reported with compounds that inhibit DNA replication, such as camptothecin, as well as in cells that have been subjected to radiation (Paulovich and Hartwell, 1995; Shao et al., 1997). The authors of these studies suggest the existence of an S-phase checkpoint that may delay progression through this phase in response to DNA damage. It is possible that the S-phase arrest seen at the higher doses of AVM-positive extracts may be related to an effect on DNA replication, but further study will be needed to determine the mechanism involved.

Flow cytometry cannot distinguish between cells in G_2 or mitosis, but DAPI staining revealed an apparent increase of cells with condensed chromosomes following AVM-positive extract exposure, suggesting that the cells are arrested in mitosis and not G2. The mechanism behind this arrest is not clear and will require further study. Cells appear to be arrested in prophase, after

chromosome condensation but before mitotic spindle assembly. While a normal spindle assembly was observed in control cells and those treated with AVM-negative extract, spindle microtubules were not observed in the majority of mitotic cells exposed to AVM-positive extracts. It is not clear whether the absence of spindle microtubules is due to a disruption of normal spindle assembly or if the cells are arrested at a point prior to spindle formation. Several compounds are known to prevent or disrupt the normal formation of the mitotic spindle, either by preventing microtubule polymerization (i.e. colchicine, vinca alkaloids, nocodazole) or stabilizing microtubules and preventing depolymerization (i.e. taxol) (Rieder and Palazzo, 1992; Johnson and Walker, 1999). However, these compounds generally tend to disrupt normal spindle organization and arrangement but do not prevent spindle assembly (Jordan et al., 1992). Colchicine and related compounds have been shown to completely inhibit formation of spindle microtubules when applied before nuclear envelope breakdown (NEB), but these cells typically progress through NEB into prometaphase where the chromosomes become dispersed throughout the cytoplasm (Rieder and Palazzo, 1992). Moasser et al. (1999) observed a similar mitotic arrest to that seen in our study by the tyrosine kinase inhibitor PD173955. They observed cells that appeared to be arrested in prophase, with condensed chromatin but no spindle apparatus. The mechanism behind this effect was not clear, but thought to be related to the inhibition of one or more protein kinases. The cell cycle arrest observed in our study could be due to a similar effect on kinase activity, or a number of other factors involved in cell cycle regulation.

Microtubules and microfilaments of interphase cells did not appear to be affected by either extract. Other compounds that induce intramyelinic edema have not been fully evaluated for their effects on microtubules or other cytoskeletal components. Zucker et al. (1989) did not examine effects on microtubules or the mitotic spindle in their study revealing G_2/M phase arrest with TET, and no other literature is available on the effects of TET on microtubules. Chow and Orrenius (1994) and Marinovich et al. (1990) found no effects of TET on F-actin in rat thymocytes and human neutrophils, respectively. Microfilaments did not appear to be affected by the extracts in our study at the applied concentration, but there is a possibility that higher doses may have had deleterious effects.

If the observed cell cycle arrest is induced by the causative agent of AVM, it is unclear how that effect may relate to the *in vivo* consequences of intramyelinic edema. The mechanisms of action of TET, hexachlorophene (HCP), and other compounds resulting in intramyelinic edema are not fully known (van Gemert and Killeen, 1998). TET, HCP, and bromethalin have been shown to affect cellular respiration, as uncouplers of oxidative phosphorylation (Moore and Brody, 1961; Cammer and Moore, 1972; Aldridge et al., 1977; Kauppinen et al., 1988; van Lier and Cherry, 1988), and TET has been shown to affect calcium homeostasis *in vitro* in several cell lines (Ade et al., 1996; Viviani et al., 1996; Kauppinen et al., 1998; Jan et al., 2002; Jiann et al., 2002; Lu et al., 2003). However, it is not yet clear how these effects may relate to the formation of vacuoles in the myelin sheath. Both TET and HCP are known to bind directly

to myelin as well as affect levels of myelin basic protein, which is though to play a role in myelin compaction (O'Callaghan and Miller, 1989; Veronesi et al., 1991; Amacher and Schomaker, 1994; van Gemert and Killeen, 1998).

As demonstrated in this study and elsewhere (Zucker et al., 1989), TET causes an arrest of cells in G_2/M *in vitro*, but the mechanism has not been elucidated and it is not known whether similar effects would be observed on cells *in vivo*. Research on the *in vivo* effects of other anti-proliferative compounds is usually focused on the treatment of cancerous cells which are rapidly dividing. After development, most cells of the nervous system, including neurons and glia, are differentiated and post-mitotic. Neural stem cells exist in the adult brain and can give rise to new neurons, astrocytes, and oligodendrocytes, but rapid proliferation of cells generally occurs only after injury (Gage, 2002). The rapid proliferation of astrocytes in response to toxic insult or other injury is well documented, but there is conflicting evidence as to whether this astrogliosis occurs in intramyelinic edema (Tripeir et al., 1981; O'Callaghan and Miller, 1989; Freeman et al., 1994; van Gemert and Killeen, 1998). Thomas et al. (1998) reported astrogliosis in one of three eagles examined with AVM in the original description of the disease, but no other reports of astrocyte involvement have been reported in AVM studies.

The effect of TET on the cell cycle does not seem to be specific to a certain cell type, as erythroleukemic (Zucker et al., 1989) and glial cells (this study) are affected in a similar manner. It is not clear whether or not similar effects of cell cycle arrest would have been seen in the other cell lines examined

for cytotoxicity in this study. Morphological observations of these cells revealed a mild rounding effect evident in both AVM-positive and AVM-negative extracts, but it is not known whether this rounding represented mitotic cells or cells undergoing apoptotic or necrotic cell death.

The C6 cell line is derived from a glioma and is astrocytic in origin, but the effect on C6 cells in this study may represent a general cytotoxic effect rather than a glial or astrocyte-specific toxicity. Established cell lines can provide indications of general cytotoxic effects as well as effects on cell division, but effects on specific cell types are usually studied using primary cultures (Mead and Pentreath, 1998). This is due to the fact that established cultures, being either transformed or developed from tumors, may no longer retain all of the original *in vivo* characteristics of that cell type (Zucco et al., 1998). Astrocyte-specific toxicity is usually studied in primary cultures; and the expression of glial fibrillary acidic protein (GFAP), an intermediate filament specific to astrocytes, has been shown to be the most sensitive indicator of astrocyte-specific toxicity (Cookson and Pentreath, 1994). C6 cells have been shown to respond similarly to primary astrocytes in cytotoxicity assays as well as measures of GFAP but only after pretreatment with dibutyryl cyclic AMP (Cookson et al., 1995).

The involvement of astrocytes in intramyelinic edema is debated. *In vivo* studies have generally found no apparent involvement of astrocytes, although one study did describe astrocytic swelling (van Gemert and Killeen, 1998). TET has been studied *in vitro* for its effects on GFAP expression in primary astrocytic cultures with conflicting results. Two studies found that while TET induced cell

death in these cultures, GFAP expression was either not affected at cytotoxic concentrations (Mead and Pentreath, 1998) or decreased in parallel with cytotoxicity (Rohl et al., 2001), while another found an increase in GFAP expression at sub-cytotoxic concentrations (Richter-Landsberg and Besser, 1994).

The other endpoints examined in this study – cytotoxicity and induction of apoptosis – did not provide significantly different results for the AVM-positive and AVM-negative extracts. Both extracts resulted in a concentration-dependent cytotoxicity in all cell lines examined. The AVM-positive extract (Parksville-A) was slightly more toxic to N2a and QNR/D cells than the AVM-negative (Potato Creek-B) extract, but it is important to remember that the extracts tested were in crude form and contained a large number of compounds associated with the vegetation and epiphytic communities present. The concentrations were normalized based on the dry weight of extracted vegetation and therefore do not provide a measure of any bioactive compounds present. It is possible that the additional presence of the AVM causative agent in the Parksville-A extract was the source of the additional toxicity; but it is equally as possible that the Parksville-A extract contained more of the cytotoxic substance or substances associated with the Potato Creek-B extract. These assays may be able to detect the AVM toxin in a more purified extract, and could be revisited when such extracts are produced.

The mechanism of cell death associated with the extracts did not appear to differ between the AVM-positive (Woodlake, Parksville-A) and AVM-negative samples (Potato Creek-A) tested, and neither appeared to induce apoptosis at the

concentrations and time points examined. The high doses of AVM-positive extracts appeared to induce a fairly acute necrotic cell death, as the number of PI stained cells in the Annexin assay and the size of the sub-G1 peak in the cell cycle assay increased with these doses up to 24 h. These doses did not produce an increase in caspase activity or Annexin V binding prior to cell death and thus appear to have undergone necrosis. Populations of cells exposed to the lower doses of AVM-positive extracts and the AVM-negative extracts did not have appreciable cell death by 24 h. It is unclear what the ultimate fate of the cells blocked in G_2/M may be. At lower doses of some extracts (SCWMA, Parksville-B, Woodlake, Petersburg) the cell populations appear to recover by 24 h. In extracts where there is a continued G_2/M phase block at 24 h (Dens Swamp, Parksville-A), it is likely that these cells proceeded to undergo apoptotic or necrotic cell death at a later time point. There are reports of both apoptotic and necrotic cell death following cell cycle arrest induced by other compounds in various cell lines (Fujikawa-Yamamoto et al., 1994; Subbegowda and Frommel, 1998; Butler et al., 2000). TET is known to induce apoptosis in primary oligodendrocytes, hippocampal and cortical neurons (Thompson et al., 1996; Stahnke and Richter-Landsberg, 2004), but the effects of other inducers of intramyelinic edema on the mechanism of cell death *in vitro* has not been evaluated. Two other organotin compounds, triphenyltin and tributyltin, have been shown induce apoptosis in several cell lines at low concentrations but result in necrosis at higher doses (Stridh et al., 1999) with triphenyltin also inducing cell

cycle arrest in G_2/M at concentrations shown to induce apoptosis (Chikahisa et al., 1998).

The cell cycle arrest as measured by flow cytometry analysis of DNA content appears to be a promising assay in the detection of the AVM toxin. However, it is not yet clear whether the mitotic block associated with the AVM-positive extracts is in fact due to the AVM causative agent and further fractionation and clean up of the crude extracts is necessary to isolate the AVM toxin for future testing. In future examinations with this assay, the 4 h time point may be sufficient to observe any significant results, as this time point appears to be the most sensitive in detecting the cell cycle disruption and as the activity associated with lower concentrations of extract is lost by 24 h. This may be due to a decrease in cell division rate at the later time points, as Rouzaire-Dubois et al. (2000, 2004) have shown that C6 cell proliferation slows as cells approach confluency. In addition, since the effects on the cell cycle observed in this study were concentration-dependent, a range of dosages should be included to minimize the chance of missing any possible activity.

The extraction procedure developed in Chapter 2 (Wiley, 2007) coupled with the discovery of the cell cycle disruption observed here provide promising tools in the future study of AVM pathology and the search for the causative agent of this disease. If the cell cycle assay can be validated as a means of detecting the AVM toxin it can then be used to guide toxin fractionation and isolation studies, provide an alternative to *in vivo* assays, and provide a model to begin investigating mechanism of action.

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CHAPTER 4

IN VIVO AND *IN VITRO* TOXICITY OF FRACTIONS PRODUCED FROM A CRUDE EXTRACT OF *HYDRILLA VERTICILLATA* AND ASSOCIATED EPIPHYTES COLLECTED DURING AN AVM EPIZOOTIC

Introduction

Avian vacuolar myelinopathy (AVM) is a neurological disease affecting bald eagles (*Haliaeetus leucocephalus*), American coots (*Fulica americana*) and other birds in the southeastern U.S. The cause of AVM is unknown, though it is suspected to be a biotoxin and has been linked to ingestion of aquatic vegetation. AVM is associated with a number of reservoirs in five southeastern states, and to date had been responsible for the deaths of over 100 bald eagles and thousands of coots (Wilde et al., 2005).

AVM is characterized and diagnosed by a spongy degeneration of the white matter of the central nervous system. Multiple vacuoles of varying severity are seen throughout the white matter of the brain and spinal cord, being particularly prominent in the optic tectum (Thomas et al., 1998). Birds with AVM may display clinical signs of neurological impairment, such as difficulty in flying, swimming, and/or walking, but not all birds with AVM lesions display clinical signs, and clinical recovery has been documented despite the persistence of lesions (Larsen et al., 2002).

AVM has been linked to the ingestion of aquatic vegetation, with several studies demonstrating induction of AVM in laboratory animals after ingestion of aquatic vegetation (*Hydrilla verticillata* and associated epiphytes) collected during AVM epizootics (Birrenkott et al., 2004; Lewis-Weis et al., 2004; Rocke et al., 2005). As demonstrated by Fischer et al. (2003), predators such as the eagle contract AVM by feeding on affected prey.

It is not yet clear what factor associated with the vegetation causes AVM, but researchers have linked the disease to an epiphytic cyanobacterium that grows on the plant material. Hydrilla that induced AVM in a mallard laboratory study contained large quantities of a novel cyanobacterial species that covered 50-90% of the leaf surface area (Birrenkott et al., 2004). Surveys of cyanobacterial and algal epiphytes revealed the consistent presence of this species, a previously unknown member of the order Stigonematales, at all known sites of AVM epizootics (Wilde et al., 2005). The cyanobacterium was present in high abundance at AVM sites, and was not observed, or observed in low abundance, at control sites. It has been hypothesized that this species is producing a neurotoxin responsible for AVM.

We have been successful at extracting the AVM toxin from vegetation collected during an AVM epizootic (Wiley, 2007, Chapter 2). This study produced a crude methanol extract that induced AVM in laboratory mallards upon exposure by oral gavage. This crude extract, along with several other extracts produced from various sites of AVM outbreaks, also induced cell cycle arrest in C6 glioma cells (Wiley, 2007, Chapter 3). The *in vitro* effects could not be

directly attributed to the AVM toxin, as the extracts were still in crude form and may have contained a variety of bioactive compounds associated with the vegetation or the epiphytic communities present. As well, extracts from vegetation collected at sites having no previous history of AVM also induced a mild cell cycle arrest.

The objectives of this study were to further fractionate a crude methanol extract to advance the isolation of the AVM toxin, as well as evaluate the effectiveness of the *in vitro* cell cycle assay at detecting the toxin. Crude extract was produced from vegetation collected during an AVM epizootic by methods developed in Chapter 2 (Wiley, 2007). Several fractions covering a full range in polarity were then isolated from this crude extract by liquid-liquid extraction as well as C18 solid phase extraction (SPE), and evaluated for toxicity by an *in vivo* chicken bioassay and the *in vitro* cell cycle analysis.

Materials and Methods

Preparation of Crude Extract

Crude methanol extracts were produced using methods developed in Chapter 2. Aquatic vegetation (*Hydrilla verticillata* and associate epiphytes) was collected from Woodlake, North Carolina 22 November 2005 during an AVM epizootic. Control hydrilla was collected 14 October 2003 from Potato Creek Embayment, Lake Marion, South Carolina, a reservoir with no known history of AVM. Hydrilla was lyophilized, blended to a coarse powder, and dry weight was recorded (Potato Creek, 605.4 g; Woodlake, 1121.1 g). Dried, powdered

vegetation was loaded evenly into two, 2 L separatory funnels and sequentially extracted with hexanes, acetone and methanol (Fisher Chemical, HPLC grade, Fischer Scientific, Hampton, New Hampshire, USA). Solvent was added to the vegetation until saturated (1-1.5 L of solvent per funnel) and the sample was mechanically extracted by shaking for five, 2 min periods over 1 h. The extract was then allowed to drain, and the sample was rinsed with a final volume of solvent (0.5 L). Drained solvents were pooled. This process was then repeated with the next solvent. Hexanes and acetone extracts were discarded. The methanol extracts were filtered through 70 mm Whatman qualitative grade filter paper (Whatman, Inc., Florham Park, New Jersey, USA) to remove particulates, and solvent was removed by a rotary evaporator (Buchi Rotavapor, Buchi, Flawil, Switzerland) with a water bath set no higher than 28°C. When dry, the extracts were re-suspended in the appropriate volume of methanol to a concentration of 10 g vegetation/ml solvent, based on the initial dry weight of the vegetation. Half of the Potato Creek crude extract was archived at -20°C for use in *in vitro* studies and half was stored at -20°C for the chick study. One-third of the crude Woodlake extract was stored at -20°C for the chick study, of which 20% was archived for *in vitro* studies. The remaining two-thirds of Woodlake crude extract was further fractionated as described below.

Preparation of Extract Fractions

Woodlake crude extract was brought to 90% methanol with deionized water and extracted 10x with hexanes to remove chlorophylls. Hexanes fractions

were pooled. The aqueous methanol fraction was then centrifuged at 3500 rpm for 10 min to remove particulates. The pellet was washed twice with 90% methanol. Supernatants were pooled and split in half. One-half of the supernatant was brought to 60% methanol and extracted 3x with methylene chloride. The remaining half of the supernatant was dried by rotary evaporator and re-suspended in 100% HPLC water for C18 SPE. Prior to running through the C18 column, the extract was filtered through 70 mm Whatman qualitative grade filter paper to remove a significant amount of particulates that did not re-suspend in the HPLC water. These particulates were re-suspended in 100% methanol and archived at -20°C for potential future testing. The dissolved fraction was run through a C18 SPE column (Varian BondElut C18 SPE column, Varian, Inc., Palo Alto, California, USA) and eluted with HPLC water; 25% methanol; 50% methanol; 75% methanol; 100% methanol; acetone; methylene chloride; and hexanes (Figure 18). All resulting fractions were concentrated by rotary evaporator and/or lyophilization and re-suspended to 10 g vegetation/ml solvent, based on the initial dry weight of the vegetation. Twenty percent of each fraction was archived at -20°C for *in vitro* testing and the remaining 80% was stored at -20°C until use in the chick study.

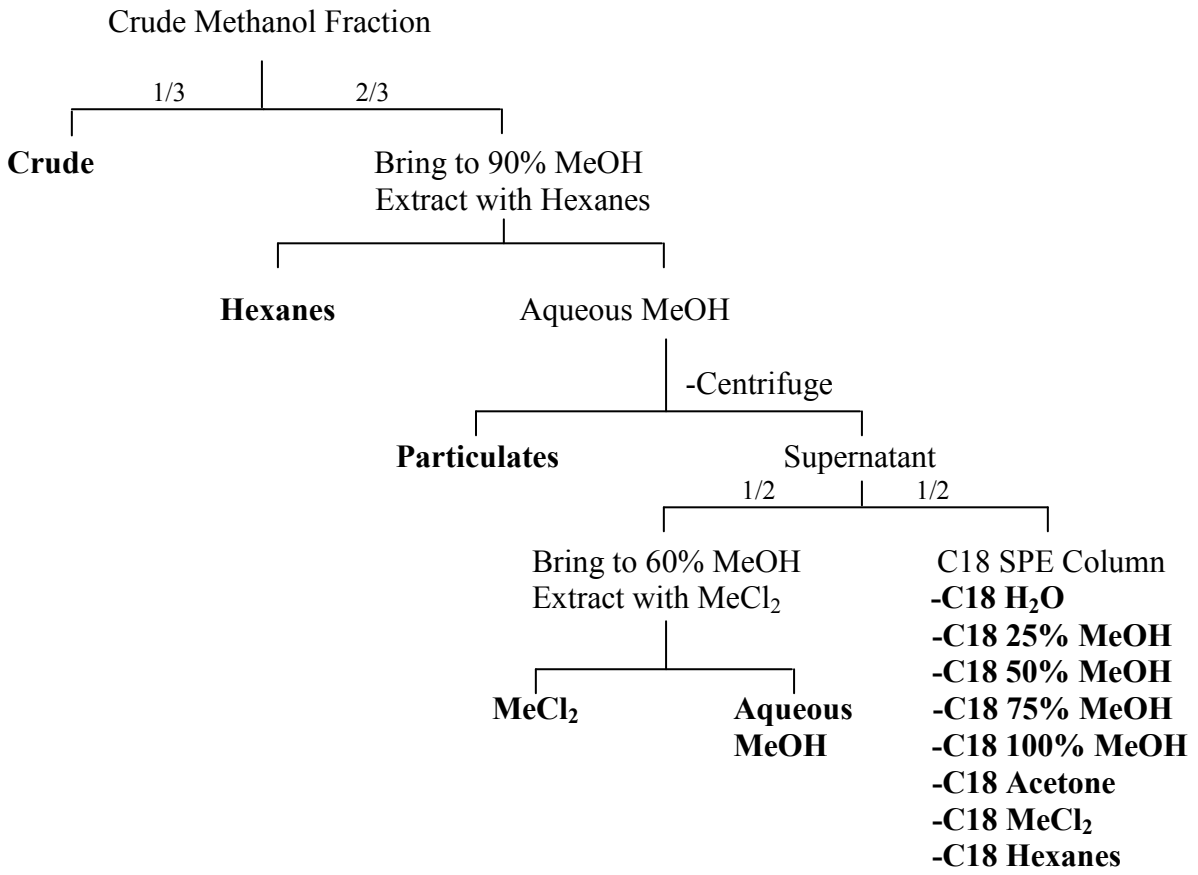


Figure 18. Fractionation of the crude methanol extract produced from vegetation collected from Woodlake, North Carolina during an AVM epizootic. MeOH=methanol. $MeCl_2$=methylene chloride.

Chick Bioassay

Forty-two, nine-day-old chicks were obtained from the Clemson University Morgan Poultry Center and transported to Godley-Snell Research Center at Clemson University. Chicks were banded with wing tags for identification and housed in a single-cage commercial brooder. Food and water were provided *ad libitum*. Chicks were acclimated four days prior to initiation of the treatment period.

Chicks were randomly assigned to experimental groups, with three birds per group. Treatments were administered by oral gavage three times a week for two weeks. Dose volumes were based on body weight at the time of dosing, at 0.00646 ml/g b.w. and 10 g hydrilla/ml. This concentration is based on the concentration given to mallards developing AVM in a previous study (Wiley, 2007, Chapter 3). Birds were observed twice daily for any sign of neurological impairment or distress. At the end of the two-week treatment period, birds were euthanized by CO_2 asphyxiation. Whole brains were removed, weighed, and fixed in 10% neutral buffered formalin. Brains were evaluated for AVM lesions at the Southeastern Cooperative Wildlife Disease Study, Athens, Georgia by methods previously described (Fischer et al., 2003).

Cell Bioassay

C6 cells were obtained from the American Type Culture Collection (ATCC). Cells were maintained at 37°C with 5% CO_2 in Kaighn's modification of Ham's F12 medium with 2mM L-glutamine modified to contain 1.5 g/L

sodium bicarbonate and supplemented with 15% horse serum and 2.5% fetal bovine serum. Cells were routinely passaged twice weekly and cells in passages 5-30 were used in assays. All cells were grown in the absence of antibiotics.

Cell cycle analysis was conducted by measuring the DNA content of cells with propidium iodide. C6 cells were plated at ~$5x10^5$/ml in 6-well plates at 2 ml per well and allowed to attach overnight. Cells were exposed to all fractions at 12.5, 50, and 100 mg/ml, as well as a vehicle control (methanol). Following exposure for 4 h, cells were harvested with trypsin-EDTA, pelleted by centrifugation at 3500 rpm for 5 min, and fixed in ice-cold 70% ethanol overnight at -20°C. Cells were then pelleted (3500 rpm, 5 min, room temperature) and stained with 10 µg/ml propidium iodide in PBS containing 10 µg/ml RNase for 1h at room temperature in the dark. Flow cytometry analysis for cell cycle distribution was conducted on a Coulter EPICS XL with excitation at 488 nm and an emission wavelength of 635 nm. A minimum of 10,000 cells were counted for each sample. The percentage of cells in G_1, S, and G_2/M phases were calculated using Multicycle software (Phoenix Flow Systems, San Diego, California, USA). The experiment was repeated with three independent culture preparations and the percentages of cells in each phase were compared by a one-way ANOVA followed by Tukey's multiple comparison test as performed by GraphPad Prism software (v 5.00, GraphPad Software, Inc., San Diego, California, USA).

Results

Chick Bioassay

No clinical signs of neurological disease or other signs of impairment or distress were noted during the study and all chicks steadily gained weight, though there were differences in weight gain among treatment groups (Figure 19). Birds dosed with Woodlake crude extract gained significantly less weight during the study than those dosed with Potato Creek crude extract, as well as those dosed with hexanes, particulates, C18 hexanes, and C18 50% MeOH fractions.

No definitive AVM-type lesions were seen in the chick brains. There were a few brains with mild white matter lesions, consisting of a low number of vacuoles located in the optic lobe, brainstem, cerebral white matter and/or cerebellar folia. These mild lesions were seen in one bird given Woodlake crude extract, two birds given the $MeCl_2$ fraction, and one bird each given the hexanes, C18 H_20, and C18 100% MeOH fractions. The bird exposed to the Woodlake crude extract had non-definitive AVM-like lesions, with diffuse tiny vacuoles present throughout the outer white matter layer of the optic lobe (Figure 20). Inflammatory lesions were also present in six brains that were indicative of a viral infection, but not associated with AVM. Brain slides are archived at the Marine Resources Research Institute, South Carolina Department of Natural Resources, Charleston, South Carolina, USA.

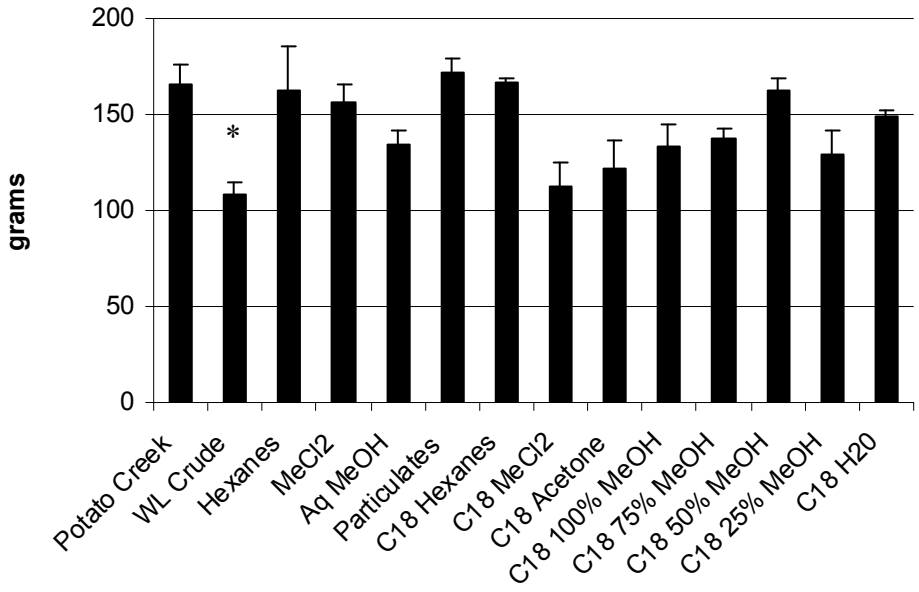


Figure 19. Average weight gain of chicks by treatment group from the initiation of the study to euthanasia. Data are represented as means ± SEM. (WL=Woodlake). Asterisk represents significant difference from Potato Creek treatment, with a p-value of ≤ 0.05.

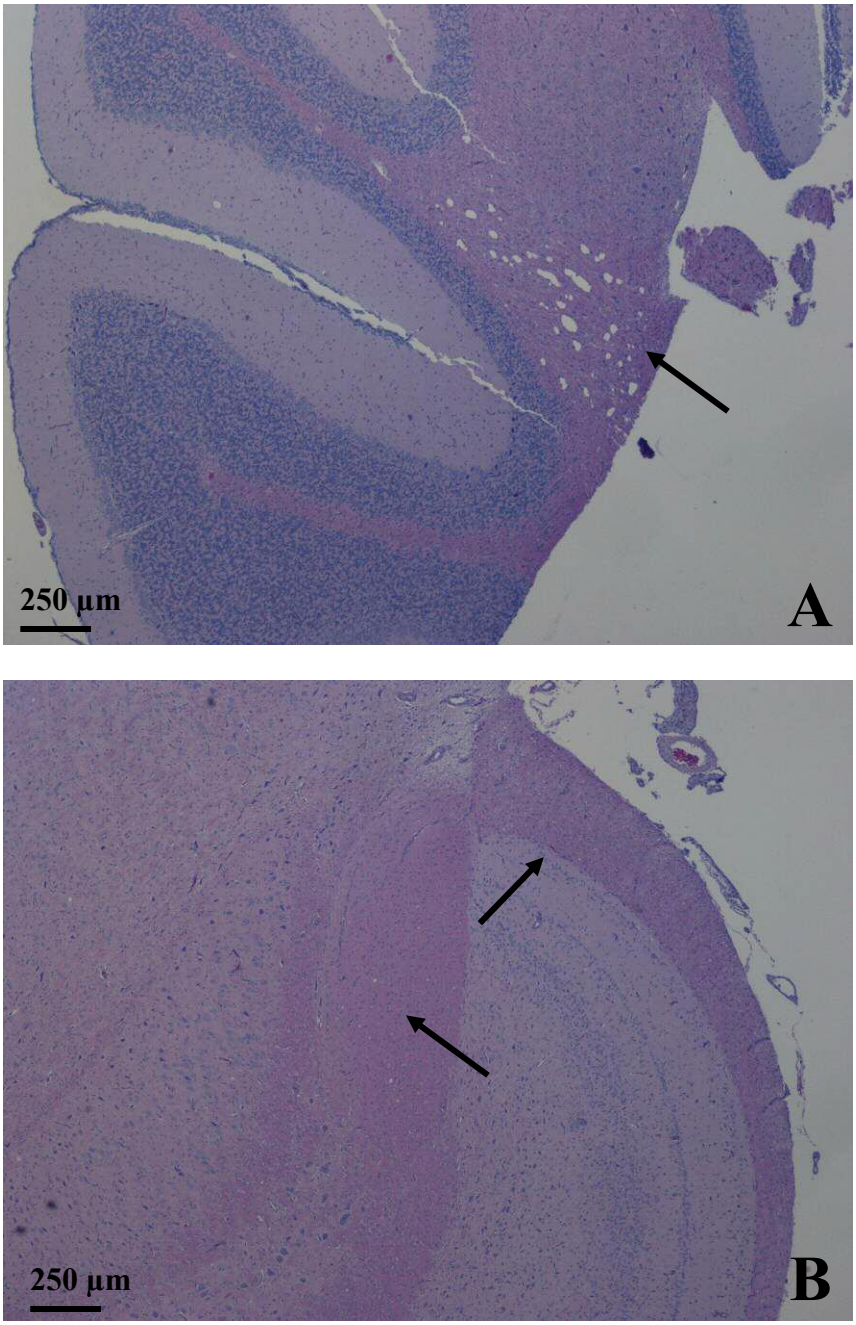
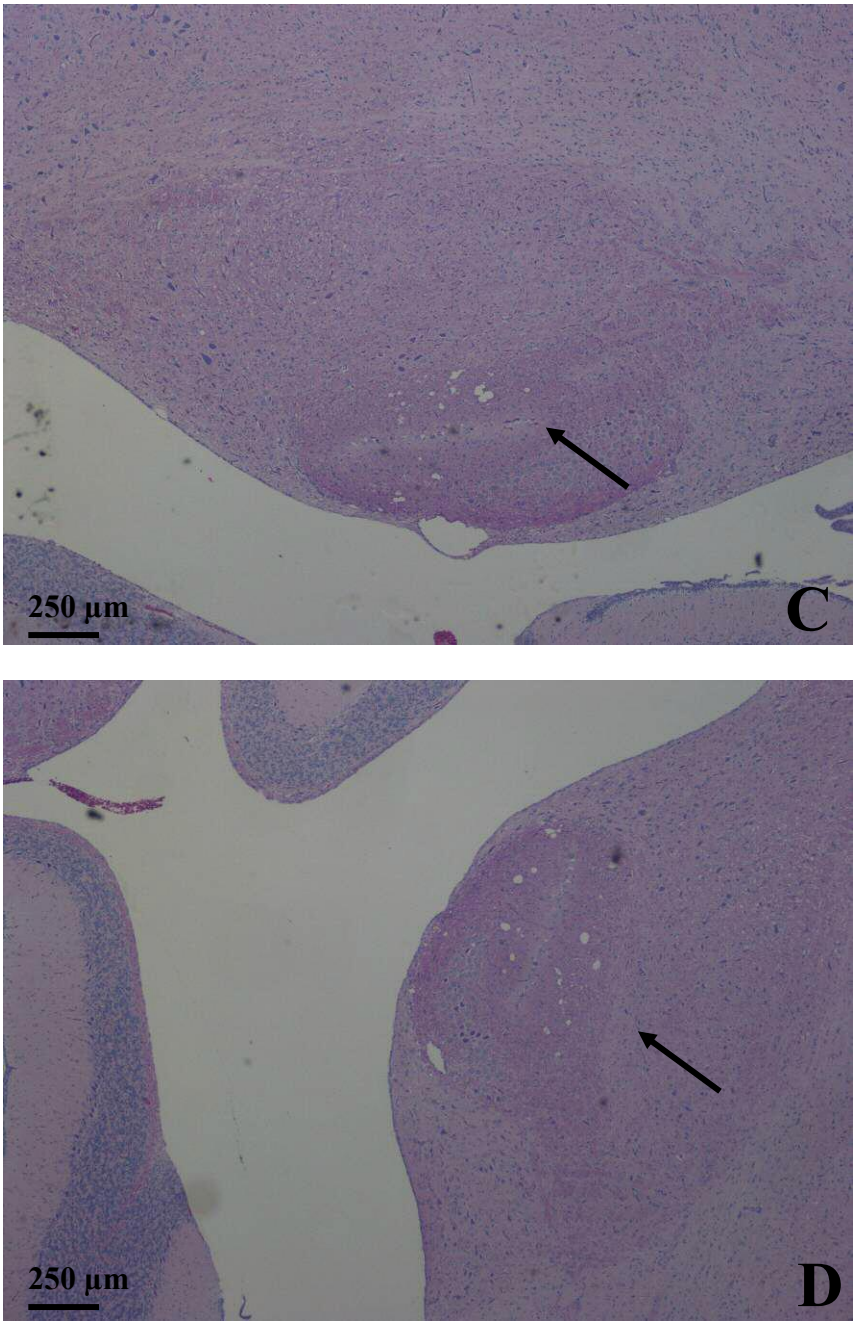


Figure 20. Mild white matter lesions in chickens exposed to a Woodlake crude extract and fractions produced from that crude extract: (A) C18 H_20; (B) Hexanes; (C); $MeCl_2$; (D) $MeCl_2$; (E) Crude; (F) C18 100% MeOH. Vacuoles can be seen in various white matter tracts (arrows).



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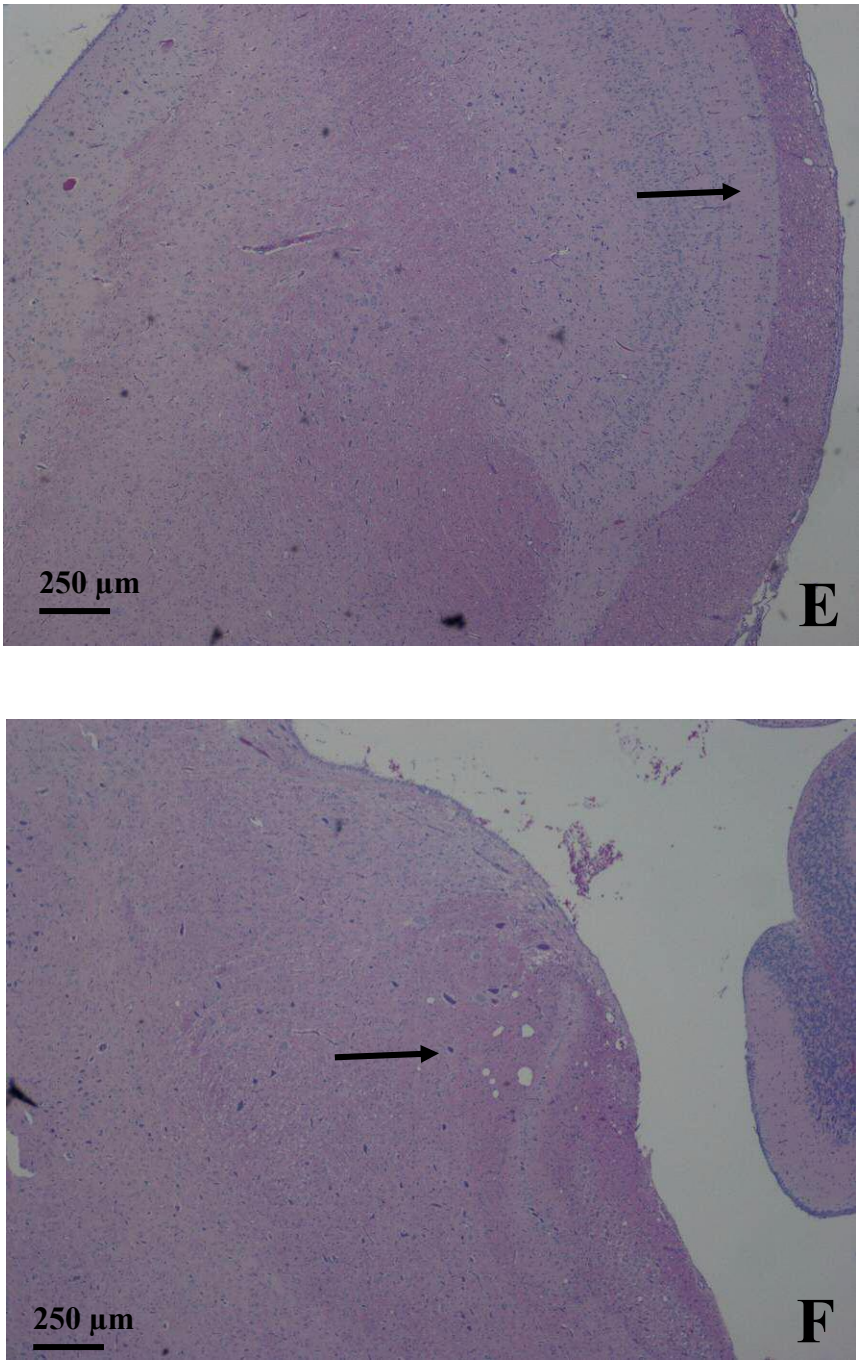

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Cell Bioassay

Significant cell cycle arrest in G_2/M phase was observed in two fractions. The percentages of cells in each phase are shown in Figure 21. The C18 100% MeOH fraction caused a significant 3-fold increase in G_2/M cells at 100 mg/ml and a significant 2-fold increase at 50 mg/ml, with concomitant decrease of G_1 cells. The $MeCl_2$ fraction caused a significant 3-fold increase in G_2/M cells at 12.5 mg/ml and a 2-fold, but not significant increase at 50 and 100 mg/ml. There were no other significant differences from control populations.

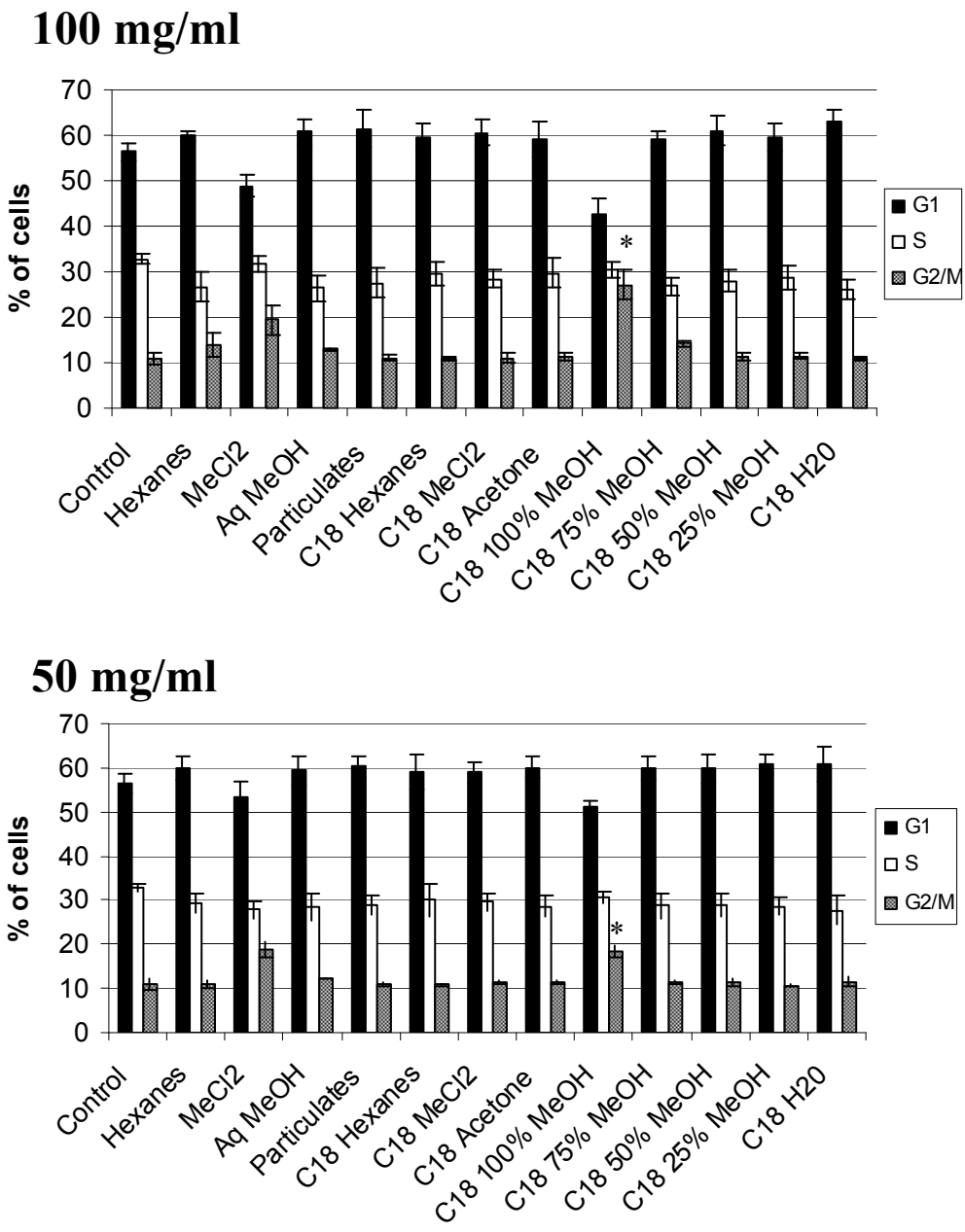
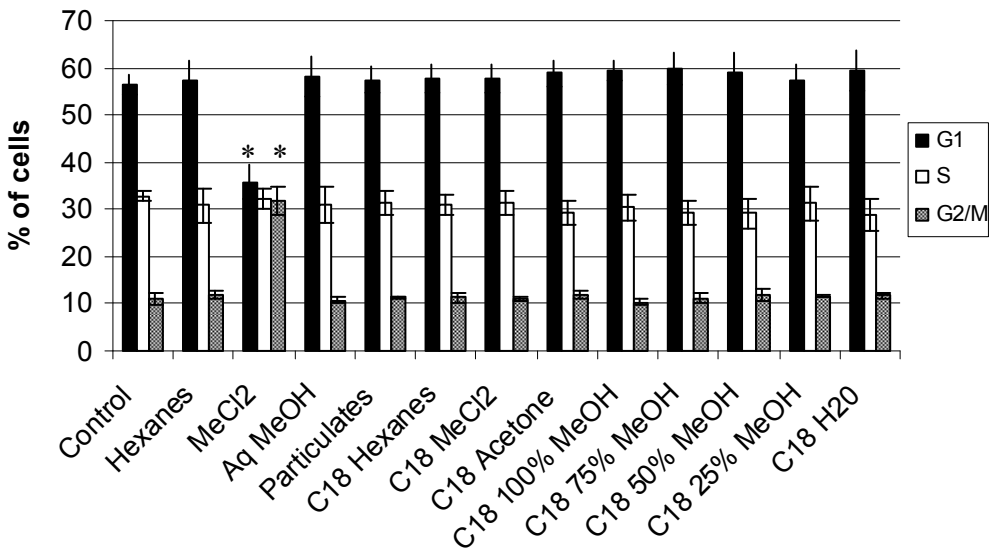


Figure 21. Cell cycle distribution of C6 cells exposed to fractions produced from the Woodlake crude extract at 100, 50 and 12.5 mg/ml and a vehicle control (methanol only). Data represent means ± SEM for three independent experiments. Asterisks represent significant difference from control, with a p-value ≤ 0.05.



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Discussion

The goal of this study was to fractionate the crude methanol extract in an attempt to further isolate the AVM toxin, thereby gaining more information on the physicochemical properties of the toxin as well as gaining further evidence that the cell cycle arrest observed with the crude extracts in Chapter 3 (Wiley, 2007) could be attributed to the toxin. *In vitro* analysis revealed significant cell cycle

arrest in two fractions of intermediate polarity: the $MeCl_2$ fraction produced from liquid-liquid extraction and the 100% MeOH fraction produced from C18 SPE. Unfortunately, the presence of the AVM causative agent in these fractions could not be confirmed because the results of the chick bioassay were inconclusive. Mild white matter lesions were present in two of three chicks brains administered the $MeCl_2$ fraction and one of three birds administered the C18 100% MeOH fraction. However, mild lesions were also seen in one of three birds administered both the hexanes fraction and the C18 H_20 fraction, which had no effect on the cell cycle.

The crude extract produced from the vegetation resulted in mild white matter lesions in only one of three birds tested. Therefore, the fact that the fractions did not produce definitive lesions is not likely due to the fractionation procedure but some other issue. The fractionation procedure did efficiently isolate the toxic activity associated with the cell cycle arrest. The $MeCl_2$ fraction induced cell cycle disruption at the same concentration as the crude extract, which also significantly arrested the cells in G_2/M at 12.5 mg/ml (Wiley, 2007, Chapter 3), suggesting that the liquid-liquid fractionation did not result in a loss of toxicity. Some toxicity associated with cell cycle arrest was lost with the C18 SPE procedure, as the C18 100% MeOH fraction did not induce cell cycle disruption below 50 mg/ml. This may due to the fact that the extract was re-suspended in 100% water prior to running through the SPE column and a portion of the extract did not dissolve in this solvent. Re-suspending the extract in 25-50% methanol in water may work more efficiently for future procedures using

C18 columns. Despite the observed *in vitro* effects, the effectiveness of the fractionation procedure at isolating the AVM toxin is unknown and will need future study.

Birds exposed to Woodlake crude extract had significantly lower weight gain on average than birds given Potato Creek extract. Decreased weight gain in AVM-affected birds has not been previously noted in other AVM studies involving chickens (Lewis-Weis et al., 2004). However, studies with triethyltin and hexachlorophene have reported decreased weight gain in rats and mallard ducklings exposed to concentrations producing intramyelinic lesions (Beirkamper and Valdes, 1982; Kung et al., 1988; Fleming et al., 1991). Since the brain results were inconclusive, it is impossible to say whether or not the decreased weight gain in this group was due to exposure to the AVM toxin. Several other treatment groups also had decreased weight gain compared to the Potato Creek group, including the aqueous methanol, C18 $MeCl_2$ and C18 Acetone groups, but those results were not significant and these fractions did not produce any toxic effects *in vitro*.

There are several possible explanations for the inconclusive results obtained in this study, including insufficient toxin concentration in the extracted vegetation, an insufficient period of exposure, an inappropriate animal model, or a combination of these factors. The vegetation extracted in this study was collected from Woodlake during an AVM epizootic in which large numbers of American coots were diagnosed with the disease (Wilde, unpubl. data). However, the vegetation was not tested by mallard bioassay prior to this study to confirm AVM

toxin presence and it is possible that the toxin was no longer present at the time of our collections (or present but at a low concentration). Coots are able to survive for several weeks after AVM lesion formation (Larsen et al., 2002), so the AVM-affected birds that were observed on Woodlake during our collections could have been exposed to the causative agent weeks earlier. Rocke et al. (2005) and Larsen et al. (2003) failed to induce AVM in laboratory mallards after exposure to hydrilla collected from Woodlake while affected coots were present. There may be a short window of toxin production and persistence in these reservoirs that is not directly correlated with the presence of affected birds. It is also possible that the toxic agent is not evenly distributed throughout the vegetation and the samples that were collected in these studies happened to contain low concentrations.

Another possible explanation for the inconclusive results in this study is that the exposure time was insufficient to produce effects. Previous laboratory and field studies have shown that birds can develop lesions within two weeks of exposure to the causative agent of AVM, with five days being the earliest known onset. However, this data was discovered only after the birds displayed clinical signs of AVM (Rocke et al., 2002; Wiley, 2007, Chapter 1) or after birds were euthanized due to unrelated reasons, as with the bird whose wing was broken in the mallard extract study (Wiley, 2007, Chapter 2). The only laboratory study undertaken with the goal of exploring the onset of AVM lesion formation was not successful since none of the birds developed AVM (Wiley, 2007, Chapter 1). Therefore, it is not clear whether all birds developing AVM experience quick lesion onset after an acute exposure, or whether some birds may develop the

disease after more chronic, low level exposure to the causative agent. Studies with triethyltin and hexachlorophene describe a concentration-dependent timing of vacuole formation in mice and rats in acute studies, with a single i.p. or i.v. injection and vacuole formation observed up to 30 h following exposure (Jacobs et al., 1977; Tripier et al., 1981). Both acute and chronic experiments have been conducted with these chemicals, but the time of lesion formation in more chronic experiments has not been evaluated.

A final possibility concerning the absence of definitive AVM lesions may be that the chickens are not a suitable animal model for studying AVM toxicity. Chickens were selected as the animal model for this research due to their smaller body weight and lower tendency to regurgitate after gavage compared to adult mallards, which have been the standard animal model used in our laboratory. Chickens are also more readily available and easy to obtain than mallards. Adult mallards were used as the animal model in the previous study involving the gavage of crude vegetative extract (Wiley, 2007, Chapter 2). This study was successful at inducing AVM lesions in these birds, but required extensive preparation time due the large quantities of vegetation that had to be lyophilized and extracted. In addition, mallards frequently regurgitated the administered material after gavage. Although the birds did subsequently develop AVM lesions, it was felt that an animal model less apt to regurgitate would be more appropriate. Young chickens had been used successfully as animal models in a previous AVM study (Lewis-Weis at al., 2004), and it was thought that these birds would provide a suitable model for this research. However, while Lewis-Weis et al. revealed

that chickens are susceptible to AVM, the relative sensitivity of chickens to the AVM agent compared to other birds is unknown.

Any or all of the above mentioned factors could be responsible for the failure to induce AVM lesions in the chicks. Unfortunately there are still many unanswered questions concerning this disease and we can only speculate as to which variables may have played a role. Although the fractionation procedure used in this study successfully isolated the *in vitro* toxicity associated with the extracts, these effects cannot be attributed to the AVM toxin due to the absence of definitive lesions in the chicks. Further studies are therefore needed to validate both the fractionation procedure and the *in vitro* assay.

It is recommended that future experiments should be conducted for at least four weeks, with vegetation that is first confirmed to contain the AVM toxin by mallard bioassay. Although these measures will increase the time and cost involved for both extract preparation and the animal trials, they will help eliminate some of the frustrating questions that have arisen with this study. The use of another animal model such as mallards should also be considered, although the problems associated with doing such a study with mallards (amount of vegetation, regurgitation concerns, and availability) will have to be taken into account.

The potential benefits of a successful fractionation procedure and validated *in vitro* model to AVM research are undeniable. Toxin isolation and characterization depend upon this research, as do mechanism of action studies.

Hopefully the results obtained in this study and the issues that have arisen in interpreting those results will aide future research efforts in this area.

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CHAPTER 5

ORAL ADMINISTRATION OF AN EXTRACT OF *HYDRILLA VERTICILLATA* CONTAINING THE ETIOLOGIC AGENT OF AVM TO A MOUSE MODEL

Introduction

Avian vacuolar myelinopathy (AVM) is a neurological disease primarily affecting bald eagles (*Haliaeetus leucocephalus*) and American coots (*Fulica americana*) in the southeastern U.S. The cause of AVM is unknown, though it is suspected to be a biotoxin and has been linked to ingestion of aquatic vegetation. AVM is associated with a number of reservoirs in five southeastern states, and to date had been responsible for the deaths of over 100 bald eagles and thousands of coots (Wilde et al., 2005).

AVM is characterized and diagnosed by a spongy degeneration of the white matter of the central nervous system. Multiple vacuoles of varying severity are seen throughout the white matter of the brain and spinal cord, being particularly prominent in the optic tectum (Thomas et al., 1998). Birds with AVM may display clinical signs of neurological impairment, such as difficulty in flying, swimming, and/or walking, but not all birds with AVM lesions display clinical signs, and clinical recovery has been documented despite the persistence of lesions (Larsen et al., 2002).

AVM has been linked to the ingestion of aquatic vegetation, with several studies demonstrating induction of AVM in laboratory animals after ingestion of aquatic vegetation (*Hydrilla verticillata* and associated epiphytes) collected during AVM epizootics (Birrenkott et al., 2004; Lewis-Weis et al., 2004; Rocke et al., 2005). As demonstrated by Fischer et al. (2003), predators such as the eagle contract AVM by feeding on affected prey.

It is not yet clear what factor associated with the vegetation is the source of AVM, but researchers have linked the disease to an epiphytic cyanobacterium that grows on the plant material. Hydrilla that induced AVM in a mallard laboratory study contained large quantities of a novel cyanobacterial species that covered 50-90% of the leaf surface area (Birrenkott et al., 2004). Surveys of cyanobacterial and algal epiphytes revealed the consistent presence of this species, a previously unknown member of the order Stigonematales, at all known sites of AVM epizootics (Wilde et al., 2005). The cyanobacterium was present in high abundance at AVM sites, and was not observed, or observed in low abundance, at control sites. It has been hypothesized that this species is producing a neurotoxin responsible for AVM.

The list of susceptible species is currently confined to birds, and in addition to bald eagles and coots, includes mallards (*Anas platyrhynchos*), ring-necked ducks (*Aythya collaris*), buffleheads (*Bucephala albeola*), Canada geese (*Branta canadensis*), great-horned owl (*Bubo virginianus*), and killdeer (*Charadrius vociferus*). Red-tailed hawks (*Buteo jamaicensis*) and domestic

chickens (*Gallus domesticus*) have also been shown to be susceptible in laboratory studies.

AVM lesions have never been confirmed in mammals during AVM epizootics, though there has been anecdotal evidence of possible clinical signs in a beaver (*Castor canadensis*) (Lewis-Weis et al., 2004). Several mammals were collected during AVM outbreaks on J. Strom Thurmond Lake, a reservoir located on the South Carolina/Georgia border, over the last few years, including beavers, raccoons (*Procyon lotor*) and a gray fox (*Urocyon cinereoargenteus*). Definitive AVM lesions were not found in their brain tissue, despite the fact that coot tissue was discovered in the stomach contents of one raccoon and the fox (Fischer et al., 2006).

There have been several laboratory studies to examine mammalian susceptibility to AVM. Swine failed to develop lesions when fed AVM-affected coot tissue, though the authors note that the duration of exposure and quantity of ingested material may not have been sufficient to induce disease (Lewis-Weis et al., 2004). Mice fed hydrilla collected during AVM events also failed to develop lesions (Rocke et al., 2005; Birrenkott, unpubl. data); however, these animals did not appear to consume much, if any, of the vegetation in at least one study (Birrenkott, unpubl. data). It is also possible that the vegetation may not have contained the causative disease agent in sufficient quantities to induce disease.

Due to the potential health implications for humans consuming affected waterfowl or otherwise coming into contact with the AVM toxin, as well as the potential for mammalian wildlife to be affected, mammalian susceptibility to the

AVM toxin needs further exploration. We have recently been successful at extracting the AVM toxin from vegetation collected during an AVM epizootic (Wiley, 2007, Chapter 2). This study produced a crude methanol extract that induced AVM in laboratory mallards upon exposure by oral gavage. The current study utilized archived extract from the successful mallard study in an attempt to induce AVM in laboratory mice. By delivering the known toxic extract by oral gavage, this study ensures that the mice are exposed to an active toxin in a concentrated form, giving a better indication of mammalian susceptibility than previous studies.

In addition to administering the crude methanol extract, a portion of the extract was further fractionated and those fractions were tested by the mouse model as well as an *in vitro* assay. Further fractionation of the crude extract and the development of an *in vitro* assay for detection of the AVM toxin would greatly benefit AVM research. The crude extract had been previously shown to induce cell cycle arrest in C6 glioma cells (Wiley, 2007, Chapter 3), and the cell cycle assay is a potentially useful means of detecting the AVM toxin. However, the cell cycle arrest could not be directly attributed to the AVM toxin as the extract was in crude form and may have contained a variety of bioactive compounds associated with the vegetation or the epiphytic communities present. Therefore, in the hopes of further validating the assay, all fractions produced from the crude extract were tested by the cell cycle assay in addition to the mouse model.

Materials and Methods

Preparation of Crude Extracts

Crude extracts were produced in a previous study (Wiley, 2007, Chapter 2). Briefly, aquatic vegetation (*Hydrilla verticillata* and associated epiphytes) was collected from an AVM-positive site (Parksville Cove, J. Strom Thurmond Lake, South Carolina, USA) and a control site with no previous history of AVM (Potato Creek Embayment, Lake Marion, South Carolina, USA) and presence or absence of the AVM causative agent was confirmed by an *ad libitum* mallard feeding study using half of the collected material (Wiley, 2007, Chapter 2). The remaining half of the vegetation was extracted as follows. Vegetation was lyophilized, blended into a coarse powder, and sequentially extracted using a series of solvents: hexanes, acetone, and methanol. Extracts were filtered and concentrated, and ten percent was archived for future use. The remaining 90% was used in a mallard gavage study to determine which extracts contained the AVM toxin. Results from the study showed that the methanol extract contained the active toxin, as all three birds exposed to the extract developed white-matter lesions consistent with AVM. The archived methanol extracts of control and AVM-positive hydrilla were used in the present study. Prior to dosing, solvent was evaporated by N_2 stream and extracts were re-suspended in a 90:10 solution of DI water:propylene glycol to a concentration of 10 g hydrilla/ml vehicle.

Preparation of Extract Fractions

The AVM-positive crude methanol extract was further fractionated for the present study (Figure 22). A portion of the crude methanol extract was brought to 90% methanol with deionized (DI) water and extracted 12x with an equivalent volume of hexanes to remove chlorophylls. Hexanes fractions were pooled. The aqueous methanol fraction was then centrifuged to remove particulates by spinning at 3600 rpm for 10 min. The pellet was washed 3x with 90% methanol and supernatants were pooled. The supernatant was brought to 60% methanol with deionized water and extracted 3x with methylene chloride. Methylene chloride fractions were pooled. Hexanes, methylene chloride, and aqueous methanol fractions were reduced by rotary evaporator, N_2 stream, and/or lyophilizer and re-suspended in a 90:10 solution of DI water: propylene glycol to a concentration of 10 g hydrilla/ml vehicle. Particulates were re-suspended in DI water to a concentration of 10 g/ml.

Mouse Bioassay

Nineteen male ICR mice (25-34 g, Harlan, Indianapolis, Indiana, USA) were received 4 April 2006 and housed three per cage by experimental group, with one extra mouse housed separately. Individuals were identified by ear punch. Animals were acclimated for six days prior to treatment and received commercial feed and water *ad libitum* throughout the study. The six experimental groups (three mice per group) consisted of the control and AVM-positive crude

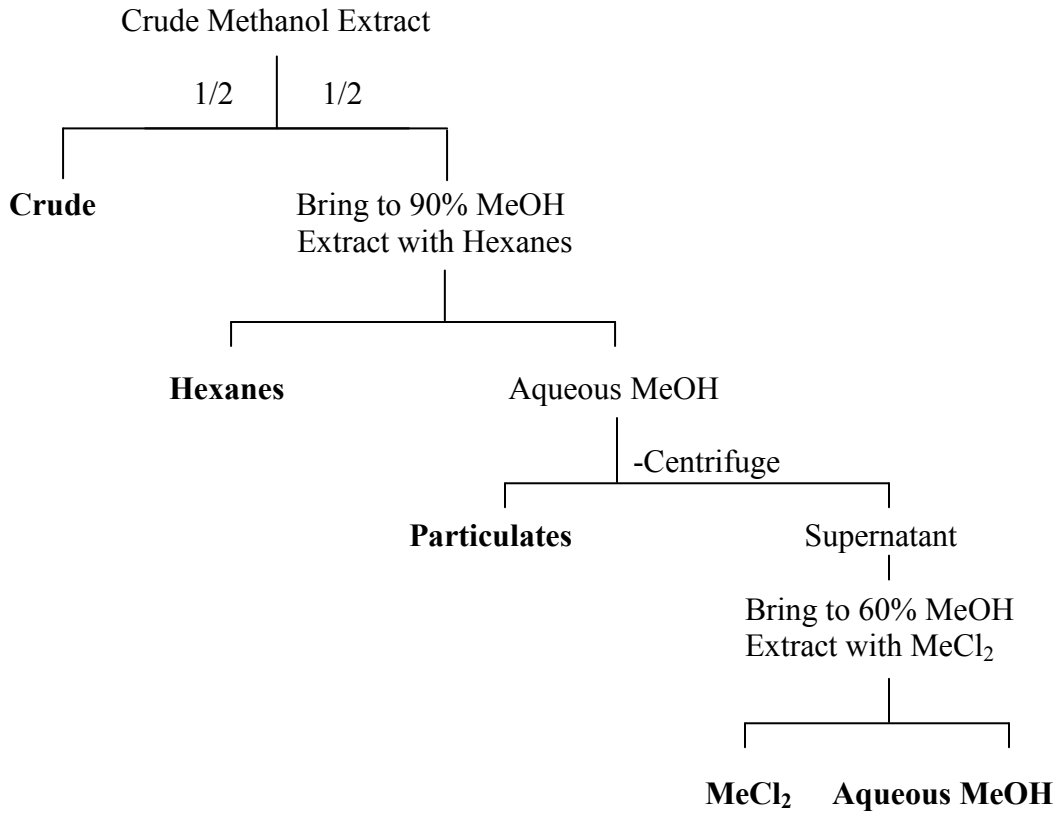

Figure 22. Fractionation of crude methanol extract containing the active AVM toxin. Four fractions result: hexanes, particulates, methylene chloride ($MeCl_2$), and aqueous methanol (MeOH).

extracts, as well as the four fractions of the AVM-positive extract: hexanes, particulates, methylene chloride, and aqueous methanol.

Extracts were delivered by oral gavage in five doses over a two-week treatment period. Dose volumes were based on body weight at the time of dosing, corresponding to the mallard extract study (0.00646 ml/g b.w. at 10 g hydrilla/ml). Mice were observed twice daily for signs of neurological

impairment or other abnormal behavior. Due to the loss of two mice in the methylene chloride group after the first dose administration (see results), the nineteenth mouse (an extra) was placed in this group. Since this mouse only received four doses, each dose was increased to give an equivalent amount as the other mice. Mice were euthanized by CO_2 asphyxiation at the conclusion of the two-week treatment period. Brains were removed, weighed, and fixed in 10% neutral buffered formalin. AVM diagnosis was conducted at the Southeastern Cooperative Wildlife Disease Study (SCWDS) by methods previously described (Fischer et al., 2003). Liver, kidney, and gastrointestinal tracts were removed and archived at -20°C.

Cell Bioassay

C6 cells were obtained from the American Type Culture Collection (ATCC). Cells were maintained at 37°C with 5% CO_2 in Kaighn's modification of Ham's F12 medium with 2mM L-glutamine modified to contain 1.5 g/L sodium bicarbonate and supplemented with 15% horse serum and 2.5% fetal bovine serum. Cells were routinely passaged twice weekly and cells in passages 5-30 were used in assays. All cells were grown in the absence of antibiotics.

Cell cycle analysis was conducted by measuring the DNA content of cells with propidium iodide. C6 cells were plated at ~$5x10^5$/ml in 6-well plates at 2 ml per well and allowed to attach overnight. Crude extracts were tested previously in Chapter 3. Fractions of AVM crude extract were tested at 12.5, 50, and 100 mg/ml. Following exposure at 4 h, cells were harvested with trypsin-EDTA,

pelleted by centrifugation at 3500 rpm for 5 min, and fixed in ice-cold 70% ethanol overnight at -20°C. Cells were then pelleted (3500 rpm, 5 min, room temperature) and stained with 10 µg/ml propidium iodide in PBS containing 10 µg/ml RNase for 1h at room temperature in the dark. Flow cytometry analysis for cell cycle distribution was conducted on a Coulter EPICS XL with excitation at 488 nm and an emission wavelength of 635 nm. The percentage of cells in G_1, S, and G_2/M phases were calculated using Multicycle software (Phoenix Flow Systems, San Diego, California, USA). The experiment was repeated with three independent culture preparations and the percentages of cells in each phase were compared by a one-way ANOVA followed by Tukey's multiple comparison test as performed by GraphPad Prism software (v 5.00, GraphPad Software, Inc., San Diego, California, USA).

Results

No signs of neurological impairment were noted during the study. Two mice ($MeCl_2$ and particulate groups) were euthanized within 2 d of the first dose administration due to swollen right sides and general lethargy. A third mouse ($MeCl_2$ group) was found dead the day following the first dose administration. Two days following the second dose administration, another mouse ($MeCl_2$ group) was found dead after appearing slightly lethargic the previous day. All four of these mice appeared to have developed infections due to gavage error. Necropsy revealed that the esophageal tract was punctured during gavage, allowing the dose solution to enter the thoracic cavity.

No white matter lesions characteristic of AVM were seen in any mouse brains. However, in 10 of the 19 mice there was unusual focal vacuolization with low numbers of vacuoles within the hippocampus, usually in the molecular layer adjacent to the pyramidal cell layer (Figure 23) and occasionally in the pyramidal cell layer. These vacuoles were seen in all experimental groups except the control, although the number of mice with vacuoles varied among the groups (Table 11). Upon consultation with several pathologists, it was determined that these vacuoles may have been an artifact of formalin fixation, though this could not be confirmed and the fact that the lesions were not seen in control mice raises suspicions. Brain slides are archived at the Marine Resources Research Institute, South Carolina Department of Natural Resources, Charleston, South Carolina, USA.

Results of the *in vitro* cell cycle analysis are shown in Figure 24. The $MeCl_2$ fraction of the AVM crude extract caused a significant increase in the number of cells present in G_2/M at 50 and 100 mg/ml. A slight but not significant increase in G_2/M cells was also seen in cells exposed to the hexanes fraction at 100 mg/ml. The control crude extract (tested in Chapter 3, Wiley, 2007), the particulate fraction and the aqueous methanol fraction did not induce cell cycle arrest.

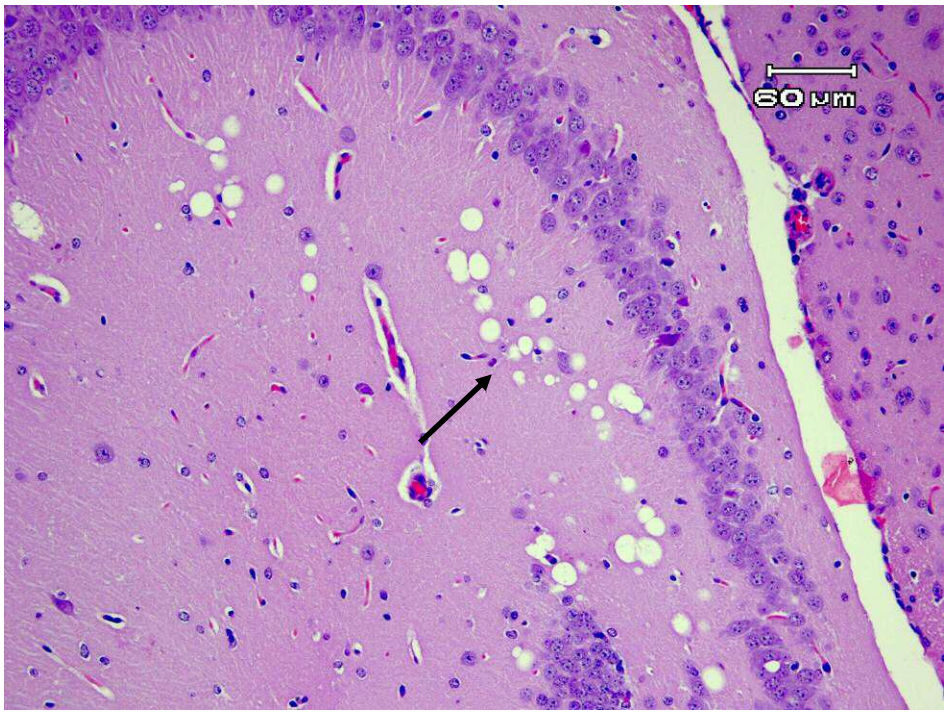

Figure 23. Light micrograph image of the hippocampus of a mouse gavaged with crude methanol extract from Parksville Cove, J. Strom Thurmond Lake, South Carolina. Unusual vacuolization is present (arrow).

Table 11. Presence of hippocampal lesions in mice exposed to extracts of *Hydrilla verticillata* and associated epiphytes collected from Potato Creek Embayment, South Carolina and Parksville Cove, J. Strom Thurmond Lake, South Carolina.

Experimental Group	Presence of lesions
Potato Creek Crude	0/3
Parksville Crude	2/3
Particulates	3/3
Hexanes	3/3
Methylene Chloride	1/4
Aqueous Methanol	1/3

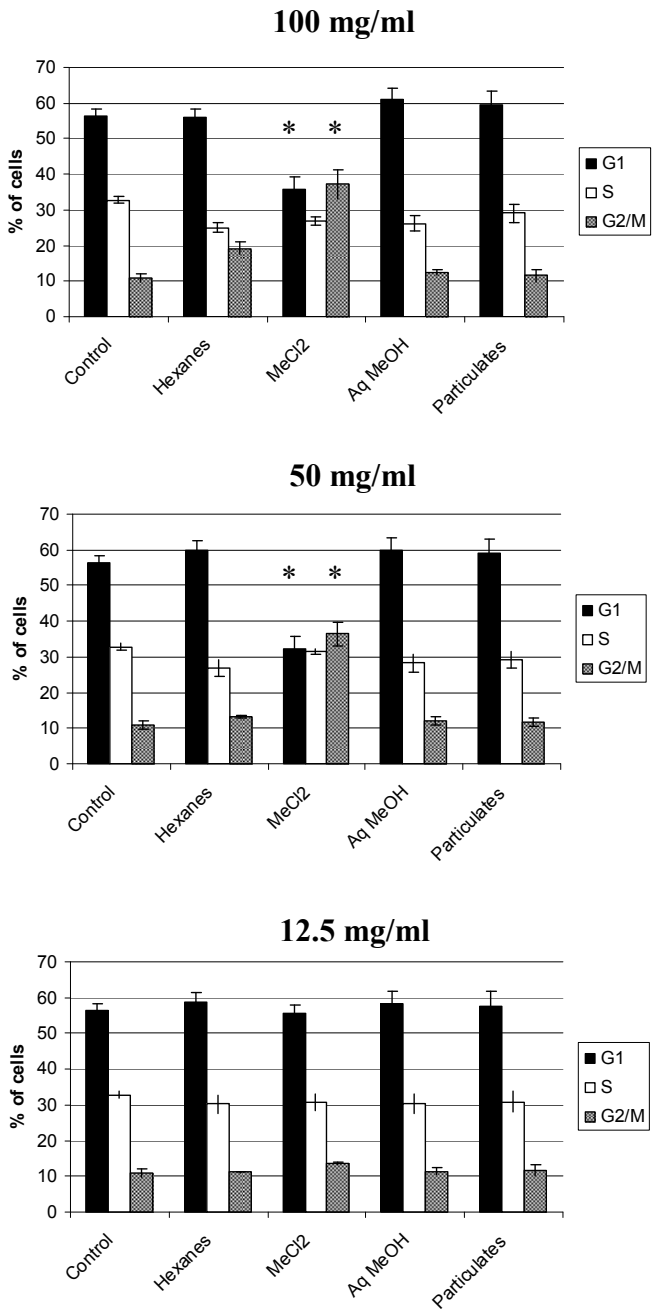

Figure 24. Cell cycle distribution of C6 cells after exposure to four fractions produced from the AVM-positive crude extract at 100, 50 and 12.5 mg/ml. Data represent means ± SEM from three independent experiments. Asterisks represent significant difference from control with a p-value ≤ 0.05.

Discussion

The goal of this study was to evaluate mammalian susceptibility to AVM by administering a known AVM-positive vegetative extract to mice. Following two weeks of exposure to the extract at a dosage which induced AVM in mallards, no mice contained white matter lesions consistent with vacuolar myelinopathy. Therefore it appears that mice are not susceptible to the causative agent of AVM at concentrations which induced moderately severe lesions in birds. These results agree with previous studies of mammalian susceptibility which found no lesion formation in mice and swine following exposure to AVM-positive vegetation and coot tissues (Lewis-Weis et al., 2004; Rocke et al., 2005; Birrenkott, unpubl. data).

While the mice did not develop characteristic AVM lesions in this study, ten of nineteen mice exposed to either the crude AVM-positive extract or the fractions produced from this extract developed unusual lesions localized to the hippocampus. These lesions may have been an artifact of the fixative process, although this conclusion is hard to fully accept due to the absence of lesions in the control mice. However, it is also unlikely that if the lesions are real they would be induced by all four fractions produced from the crude extract. The fractionation procedure produced four fractions of widely varying polarity, with the extremely non-polar hexanes fraction and the extremely polar particulate fraction (which was only soluble in water and not methanol), as well as both intermediate fractions, resulting in hippocampal lesion formation.

The fractionation procedure did isolate the *in vitro* toxicity associated with the extracts, as the $MeCl_2$ fraction induced a significant increase of cells in G_2/M following a 4 h exposure. These results are consistent with those in Chapter 4 (Wiley, 2007), in which the $MeCl_2$ fraction produced from vegetation collected from Woodlake, North Carolina during an AVM epizootic induced a significant cell cycle arrest. The Woodlake fractions were also tested by an *in vivo* chicken bioassay to confirm toxicity, but histological brain analyses for that experiment were inconclusive. Since the mice in the present study also did not develop characteristic AVM lesions, it is not known whether the observed *in vitro* effects in these studies are due to the AVM toxin or some other bioactive compound associated with the extracts.

Despite the fact that mice in this study did not develop characteristic AVM lesions, there is still a possibility that mammals could be affected by the causative agent but are less sensitive than birds. The extract dosage used in this study was based on that which produced lesions in mallards. The archived extract from the mallard study was only sufficient to administer this dosage to the mice for two weeks. It was thought that this time period would be sufficient, as several studies have now shown that the onset of disease can occur within a two week time period (Rocke et al., 2002; Wiley, 2007, Chapter 2, Chapter 3). However, the dynamics of lesion onset are not yet understood, and it is possible that an acute exposure could produce lesions if toxin concentrations are sufficient, but also that lower concentrations given over a longer time period may also result in AVM. Therefore, it is possible that a higher concentration of the extract or a longer

exposure period would have resulted in AVM lesion formation. It is also possible that while acute exposure to the AVM toxin may not result in the same pathology in mammals as in birds, chronic exposure to the toxin may prove to be harmful in some way.

While this study provides a better indication of mammalian susceptibility to AVM than previous studies, it is not conclusive evidence that mammals are not affected by the causative agent of AVM. Further study is needed to fully evaluate the toxic potential of this disease for mammalian wildlife and humans. The development of an extraction procedure for the toxin and work towards further isolation of the toxin in crude extracts will provide tools for future research. If a cleaner extract can be developed it could be administered to a mammalian model by various routes (intravenous, intraperitoneal, and/or oral) at a wide range of concentrations. It would also be wise to test the extract in acute and chronic experiments and examine effects on various parameters in addition to lesion formation, including full behavioral evaluations while the animals are live, as well as histological evaluation of other tissues and biochemical analysis on blood samples. Increasing the sample size per group beyond three animals and processing the brain tissue by a different method would also be helpful in evaluating the hippocampal lesions seen in this study.

The potential for this disease to impact mammalian wildlife and human health is evident. Mammals such as raccoons and foxes have been documented as consuming American coots (Fischer et al., 2006), and other herbivorous mammals such as beavers are exposed through their diet. Humans consume waterfowl that

are known to be affected by AVM, and the potential exposure is increased by the fact that hunting season corresponds with AVM epizootics. While the evidence so far suggests that mammals are not susceptible to this disease, further study is needed to more fully evaluate the potential effects of the AVM agent on these species.

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CONCLUSIONS

The major objectives of this dissertation were to develop extraction methods for isolating the AVM toxin from vegetative samples and to develop an *in vitro*, cell-based bioassay for detection and study of the toxin. The AVM toxin was successfully extracted from vegetative samples and methanol was shown to be a suitable solvent for this procedure. The AVM toxin is probably moderately polar and water-soluble. It is stable at -20°C for at least one year and is not extremely volatile. Further fractionation of the crude extract will be necessary to provide a more accurate and detailed description of the physical properties. I attempted to further fractionate the crude extract, but unfortunately the presence of the AVM toxin in those fractions could not be confirmed due to inconclusive results from the *in vivo* bioassays.

In vitro toxicity associated with the extracts for AVM-positive sites revealed that the toxin may arrest the cell cycle of C6 glioma cells and this effect may be an efficient measure of detecting the AVM toxin in environmental samples. However, this effect cannot yet be attributed to the AVM toxin, as the extracts tested were in crude form and may have contained other bioactive substances. The toxic activity associated with cell cycle arrest was further isolated by fractionation of the crude extracts, but could not be confirmed as the AVM toxin due to the inconclusive *in vivo* results.

In vivo bioassays remain the only method of confirming the AVM toxin in environmental samples. The cell cycle assay may provide a suitable *in vitro* assay for toxin detection, but it must first be validated through the use of parallel *in vivo* studies. The challenges that occur with *in vivo* studies, including the choice of an appropriate animal model and time period of exposure, must be resolved before a successful *in vitro* method can be confirmed. Mallards appear to be the most reliable model, but require an extensive amount of sample material to conduct the assays and are also difficult to acquire. Chickens are more readily available, and young chicks require much less sample material, but it is unclear whether they are as susceptible to the AVM agent as mallards. A parallel study using both mallards and chicks would be useful in determining the susceptibility differences of these species. The time period of exposure necessary to induce toxicity is also still unknown, but it is recommended that subsequent *in vivo* assays include at least a four-week exposure period until this variable is more fully understood.

The presence of AVM-affected birds on a reservoir is not sufficient evidence that the AVM toxin will be present in collected vegetation. Therefore, vegetation collected for future toxin isolation procedures should be tested by an *in vivo* bioassay to confirm AVM toxin presence prior to conducting toxin extraction. While this will add an extra step in the research process, it will prevent the waste of time and resources in preparing extracts of vegetation that may not be toxic. It is also recommended that vegetation be collected as soon as AVM-affected birds are documented on the reservoirs to have a better chance of collecting material containing the active toxin.

The issue of mammalian susceptibility is not fully resolved. Mice exposed to the AVM toxin by gavage in this research did not develop characteristic AVM lesions, providing further evidence that mammals are not susceptible. However, unusual hippocampal lesions were present in several mice exposed to these extracts. While these lesions may be artifactual, additional experiments are needed to further evaluate a possible causative link between the AVM toxin and the presence of these lesions.

AVM continues to impact eagle and coot populations in the Southeast. Management and mitigation of AVM depends upon continued research efforts towards identifying the source of this disease. The extraction methods developed here, as well as the preliminary work at identifying an *in vitro* model for AVM, will greatly benefit the continued search for the causative agent. Further efforts at isolating the toxic agent and validating the *in vitro* assay are needed to continue this valuable line of research.